STUDIES ON THE *IN VITRO* CULTIVATION OF THE INTRAERYTHROCYTIC STAGES OF *THEILERIA ANNULATA*, *THEILERIA PARVA* AND *BABESIA BOVIS*

By

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This thesis is dedicated

to my brother,

Christopher C. Conrad,

with love and gratitude.

I, Patricia Ann Conrad, declare that

this thesis was composed by me,

and that the work described

therein was my own.

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ABSTRACT

Studies were conducted on the haemoprotozoal parasites Theileria annulata, Theileria parva and Babesia bovis maintained in stationary cultures of bovine erythrocytes.

The effect of different factors on the growth in vitro of T. annulata was evaluated. These factors included concentrations and methods for the preparation of bovine erythrocyte suspensions, reduced oxygen tensions, complex tissue culture media, sera, medium supplements and bovine aortic endothelial monolayers. In repeated experiments with two strains of T. annulata, intraerythrocytic multiplication occurred by division into quadruplet forms. Transmission electron microscopic studies showed that quadruplet forms resulted from schizogony and that these parasites had the ultrastructural features of merozoites.

The number of erythrocytes with four T. annulata merozoites, identical to the quadruplet forms seen in cultures, increased during the initial parasitaemic rise in two splenectomized carrier calves and in two splenectomized calves that had been inoculated with parasitized erythrocytes. Analysis of the incidence and distribution of parasitized erythrocytes in the calves indicated that intraerythrocytic division into four was the primary mode of multiplication for T. annulata.

A simple technique for the in vitro isolation of peripheral blood lymphocytes was tested and proved to be more sensitive than standard diagnostic techniques for detecting the persistence of the intralymphocytic schizonts of T. annulata in chronic carrier cattle.

Light and transmission electron microscopic studies showed that in stationary erythrocyte cultures, T. parva multiplied by the same schizogonous process as T. annulata, to form four intraerythrocytic merozoites. In T. parva cultures a maximum of 20-30% of the parasitized erythrocytes contained quadruplet forms by day 6-10 in vitro whereas an incidence of 40-60% was seen in comparable cultures of T. annulata. Failure of T. parva and T. annulata merozoites to reinvade erythrocytes in vitro prevented the establishment of continuous cultures.

Continuous cultures of two B. bovis isolates, from Mexico and South Africa, were established and the effect on parasite growth of different complex media and concentrations of sera was evaluated. An invasion assay was used to compare the in vitro infectivity for fluorescein-stained erythrocytes of B. bovis, T. annulata and T. parva merozoites.

Studies on the incorporation of tritiated nucleic acid precursors by B. bovis in vitro showed that the purines, hypoxanthine, adenosine, adenine and guanosine were incorporated to a greater extent than the pyrimidines, uridine and cytidine. There was no apparent uptake of thymidine by either of the two B. bovis isolates in vitro.

ABBREVIATIONS

In Text and Tables

AS	autologous serum
BAE	bovine aortic endothelial monolayer
CTVM	Centre for Tropical Veterinary Medicine
ECF	East Coast fever
FBS	foetal bovine serum
GSH	reduced glutathione
M199/20 FBS	Medium 199 with 20% foetal bovine serum
NBS	normal bovine serum
PBS	phosphate buffered saline - calcium and magnesium free
S.D.	standard deviation
t.e.	tick equivalent

On Electronmicrographs

DM	developing merozoite
FV	food vacuole
HC	host cell
HN	host nucleus
IM	inner membrane segment
MC	microneme
ME	merozoite
M	double membraned organelle (mitochondrion?)
MT	microtubules
N	nucleus
PM	plasmalemma
R	rhoptry
RB	residual body
RI	ribosomes

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INTRODUCTION

Mortality and reduced productivity caused by the protozoal parasites, Theileria annulata, Theileria parva and Babesia bovis have impeded cattle improvement programmes in many tropical countries (Purnell, 1978; Dolan and Young, 1981; McCosker, 1981). Systems for the in vitro cultivation of these pathogenic haemoparasites have been utilised in research projects aimed at developing effective vaccines against bovine theileriosis and babesiosis (Brown, 1980; Ristic and Levy, 1981).

The most pathogenic stage of Theileria, the intralymphocytic macroschizont, can be maintained indefinitely in vitro as a consequence of the unique relationship between the parasite and the host lymphoid cell. Infection with Theileria induces bovine lymphocytes to transform to lymphoblastoid cells which subsequently divide in synchrony with the intracellular parasites (Hulliger, 1965; Irvin, Ocama and Spooner, 1982). Cultures of theilerial macroschizonts can be established with lymphocytes isolated from blood or tissues of infected cattle and by the in vitro infection of normal bovine lymphocytes with tick derived sporozoites (Brown, 1979a).

In the past 20 years, in vitro cultivation techniques have markedly facilitated basic biological and immunological research on Theileria. Cultivated macroschizont-infected lymphoblastoid cells have served as the basis for vaccines against T. annulata and T. parva (Pipano, 1981; Brown, 1981).

Presently, the macroschizont is the only stage of Theileria that has been propagated continuously in vitro. The microschizont

stage, in which intralymphocytic merozoites develop, is only occasionally seen in Theileria-infected lymphoblastoid cell lines. Reported techniques for inducing the formation of merozoites which could infect erythrocytes added to T. annulata and T. parva cultures (Hulliger, Brown and Wilde, 1966; Danskin and Wilde, 1976a,b; Jongejan, Perié, Franssen and Uilenberg, 1980) have not been successfully reproduced in other laboratories (Shad-del, 1977; Uilenberg and Pipano, 1981). Additionally, methods for the continuous cultivation of the intraerythrocytic piroplasm stage of Theileria have not been reported.

The development of a system for the continuous propagation of the human malarial parasite, Plasmodium falciparum, by Trager and Jensen (1976), was a major breakthrough in the cultivation of intraerythrocytic protozoa. Following in the footsteps of this discovery, methods have been developed for the continuous cultivation of Babesia bovis (Erp, Gravely, Smith, Ristic, Osorno and Carson, 1978; Levy and Ristic, 1980). The successful cultivation of these parasites encouraged the undertaking of this research project with the intent of developing a system for the long-term in vitro cultivation of theilerial piroplasms.

The literature indicated that there has been persistent controversy concerning the ability of T. parva to multiply within the erythrocytes of infected cattle (Koch, 1905; Cowdry and Danks, 1933; Neitz, 1964; Büttner, 1967a; Barnett, 1968). T. annulata is apparently capable of intraerythrocytic multiplication, but the mode of division is still unclear (Dschunkowsky, 1952; Mehlhorn, 1982). The first questions to be asked in this study were whether the intraerythrocytic stages of Theileria multiply, and if so, by what process?

If intraerythrocytic multiplication occurred *in vivo*, the next question was whether continuous multiplication could be sustained *in vitro*? Continuous cultures of *B. bovis* were established to serve as a positive control in these *in vitro* experiments and to provide a source of parasites for use in developing techniques applicable to *Theileria*.

A continuous cultivation system for the intraerythrocytic stages of *Theileria*, if developed, would have a variety of potential applications. The ultrastructural and biochemical features of intraerythrocytic multiplication could be investigated more thoroughly *in vitro* than is possible *in vivo*. Microtitre cultures of *Theileria*-infected erythrocytes could be utilised for preliminary drug screening and immunological studies. The metabolism, antigenic structure and genetic constitution of piroplasms could also be studied if a constant source of cultivated parasites was available. In addition, progress towards an ultimate goal, to complete the life cycle of *Theileria in vitro*, would be made if a continuous cultivation system were established for the intraerythrocytic piroplasms.

CHAPTER ONE

LITERATURE REVIEW

1.1 General Features of the Parasites

Theileria annulata (Dschunkowsky and Luhs, 1904), Theileria parva (Theiler, 1904) and Babesia bovis (Babes, 1888) are primarily tick-borne haemoprotozoal pathogens of cattle. Theileria and Babesia are closely related genera being taxonomically classified in the subkingdom PROTOZOA, phylum APICOMPLEXA, class SPOROZOEA, subclass PIROPLASMIA, order PIROPLASMIDA and, respectively in the families Theileriidae and Babesiidae (Levine, 1971; Levine and the Committee on Systematics and Evolution of the Society of Protozoologists, 1980). The intraerythrocytic piroplasms of Theileria and Babesia are the developmental stages infective for the tick vectors.

T. annulata is transmitted transstadially by ixodid ticks of the genus Hyalomma, and causes a disease known as tropical theileriosis or Mediterranean Coast fever (Sergent, Donatien, Parrot and Lestoquard, 1931, 1945; Bhattacharyulu, Chaudhri and Gill, 1975; Robinson, 1982; Gautam and Dhar, 1983). Tropical theileriosis is widely distributed in the countries of southern Europe and North Africa, the Mediterranean littoral from Portugal and Morocco eastwards through the Near East, Middle East and southern Russia to the Indian subcontinent, China and the Far East (Dschunkowsky, 1948; Purnell, 1978).

The primary vector of T. parva is Rhipicephalus appendiculatus whose distribution restricts the parasite to East and Central Africa,

where it causes a disease known as East Coast fever (ECF) (Wilde, 1967; Purnell, 1977; Irvin and Young, 1980). ECF has recently been recognized as a disease complex encompassing several parasite species or sub-species of which T. parva parva, the primary sub-species in East Africa, is the most virulent (Lawrence, 1982; Uilenberg, Perié, Lawrence, de Vos, Paling and Spanjer, 1982).

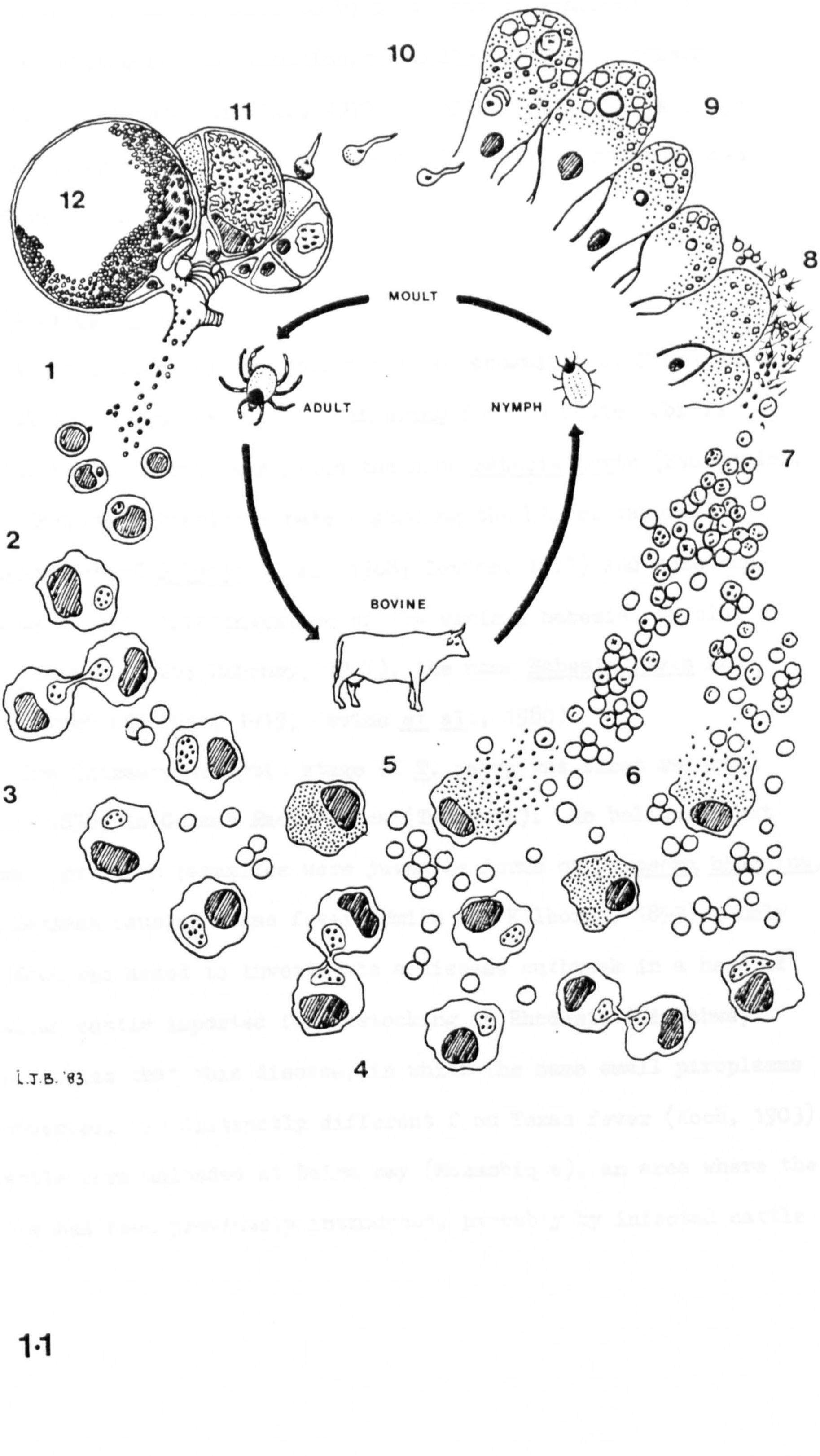
Basic features of the life cycle of Theileria are described in Figure 1.1. Studies on T. annulata and T. parva in the invertebrate host have revealed a complex development commencing with gametogony in the gut and culminating with sporogony in the salivary glands of the infected tick (Cowdry and Ham, 1932; Schein, Büscher and Friedhoff, 1975; Schein and Friedhoff, 1978; Fawcett, Büscher and Doxsey, 1982). The general biology of these theilerial parasites and the clinical features of the disease state induced in the bovine host have been reviewed by Sergent et al. (1945), Neitz (1957), Barnett (1968, 1977), Uilenberg (1981) and Irvin and Mwamachi (1983).

The extensive geographic distribution of B. bovis is dependent on the presence of the tick vectors, most notably Boophilus microplus (Purnell, 1981). The parasite is transmitted transovarially, and not transstadially, by the one host Boophilus ticks (Mahoney and Mirre, 1979). The development of B. bovis (synonym Babesia argentina) in B. microplus was described by Riek (1966) and was subsequently investigated by electron microscopy (Potgieter and Els, 1976; Potgieter, Els and Van Vuuren, 1976).

The infective forms of B. bovis, inoculated by the larval tick, invade bovine erythrocytes and transform into small (1.0-2.5 μ)

Figure 1.1 General life cycle of Theileria annulata and Theileria parva.

Sporozoites in the saliva of feeding ticks are injected into the bovine host and invade lymphocytes (1). Multiple nuclear divisions occur as the uninucleate trophozoite develops to a macroschizont (2). The presence of the parasite induces the host cell to transform to a lymphoblast that then divides in synchrony with the macroschizont (3). Synchronous host-parasite division continues (4) until merozoites begin to form at the microschizont stage (5). Rupture of the host lymphoblast releases hundreds of merozoites (6) which invade erythrocytes and develop as piroplasms (7). The subsequent ability of the intraerythrocytic piroplasms to multiply will be a topic of this thesis. After parasitized erythrocytes are ingested by an immature tick, sexual development, with the ultimate fusion of macrogametes and microgametes, may occur in the gut lumen (8). Zygotes in the gut epithelium (9) enlarge and develop into motile kinetes (10). Kinetes migrate through the gut wall, into the haemocoel and to the tick's salivary glands (11). Sporogony begins in the salivary gland acini after kinete invasion and culminates in the formation of infective sporozoites when the tick feeds in the next instar (12).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pyriform or round trophozoites (Mahoney, 1977). Asexual multiplication occurs in the erythrocyte by a process of schizogony or budding resulting in the formation, generally, of two merozoites (Riek, 1968; Potgieter and Els, 1979). Unlike Theileria, a stage of exoerythrocytic multiplication in the bovine host has not been identified for B. bovis.

1.2 Historical Review

A small intraerythrocytic parasite recognized by Babes (1888) in the blood of Rumanian cattle suffering from an acute febrile haemoglobinuric disease was given the name Babesia bovis (Starcovici, 1893). Despite extensive debate regarding the higher taxonomic classification of Babesia (Riek, 1968; Levine, 1971) and some confusion as to the differentiation of the various babesial species of cattle (Wenyon, 1926; Mahoney, 1977), the name Babesia bovis has been retained (du Toit, 1919; Levine et al., 1980).

The intraerythrocytic stage of T. parva was first reported by Koch (1898) in German East Africa (Tanzania). He believed that the small pyriform parasites were juvenile forms of Pyrosoma bigemina, the piroplasm causing Texas fever (Smith and Kilborne, 1893). Only after Koch was asked to investigate a disease outbreak in a herd of Australian cattle imported for restocking in Rhodesia (Zimbabwe) did he realize that this disease, in which the same small piroplasms were observed, was distinctly different from Texas fever (Koch, 1903). The cattle were unloaded at Beira Bay (Mozambique), an area where the parasite had been previously introduced, probably by infected cattle

from German East Africa (Tanzania). The susceptible cattle acquired the parasite while grazing on the coastal veldt and carried the disease inland to Umtali from where it spread throughout Rhodesia (Koch, 1903; Theiler, 1904). The Rhodesian fever spread into the Transvaal with the movement of cattle both from Beira and from Delagoa Bay (Mozambique). In the Transvaal, Theiler identified the parasite as a new species, Piroplasma parvum and proposed the name East Coast fever for the disease which devastated cattle herds in Southern Africa.

The new piroplasm differed from the Texas fever parasite in that it did not effect severe erythrocytic destruction, could not be transmitted to susceptible cattle by the inoculation of blood and was naturally transmitted by the brown tick, R. appendiculatus (Lounsbury, 1903, 1904; Theiler, 1904). Soon after the discovery of T. parva, then recognized as Piroplasma parvum, morphologically similar intraerythrocytic parasites were reported as the cause of tropical theileriosis in Transcaucasia, Piroplasma annulatum (Dschunkowsky and Luhs, 1904), and benign theileriosis in South Africa, Piroplasma mutans (Theiler, 1906). Bettencourt, França and Borges (1907) proposed that these three Piroplasma parasites be placed in a new genus, Theileria, with the bacilliform piroplasms which they saw in a fallow deer and those described by Miyajima and Shibayama (1906) in Japan. The Japanese parasite was probably a member of what is now regarded as the Theileria sergenti/orientalis group (Uilenberg, 1981).

The taxonomic classification of Theileria has, from the onset, been controversial and was reviewed at different times by Wenyon

(1926), Meyer (1930), Neitz (1956, 1959) and Barnett (1968). The three focal points of the debate, relevant to this thesis, are presented from a historical perspective as they emphasize the progress which has been made in understanding the developmental stages in the life cycle of Theileria species of cattle.

The points of interest are:

1. Exoerythrocytic parasite multiplication in the lymphoid cells of the bovine host.
2. Multiplication of the piroplasms in bovine erythrocytes.
3. Development of sexual stages within bovine erythrocytes or in the gut of the vector tick.

Exoerythrocytic parasites were originally identified by Koch (1905) within cells of the spleen and lymph nodes of cattle with ECF. The earliest comprehensive description of the development of T. parva in cattle was presented by Gonder (1910, 1911a,b) who argued that the presence of the intralymphocytic stages should characterize the genus Theileria. According to Gonder, T. parva was the only species of Theileria in East Africa, until exoerythrocytic (macroschizont) stages were identified in the lymphoid cells of cattle with benign theileriosis (Viljoen and Martinaglia, 1928; Theiler and Graf, 1928). The causative parasite became known as Theileria mutans after previous classifications in the genera Piroplasma (Theiler, 1906), Babesia (Gonder, 1910; Wenyon, 1926) and Gonderia (du Toit, 1919).

Theileria annulata was recognized as a distinct species in the early taxonomic classifications (Bettencourt et al., 1907; França, 1909; du Toit, 1919). However, this position was disputed by those who argued that the disease described by Dschunkowsky and Luhs (1904) was a mixed infection of Babesia bigemina, Anaplasma marginale and

T. mutans (Gonder, 1911a; Brumpt, 1923) or the parasite was a strain of T. parva (Wenyon, 1926).

Dschunkowsky (1927) defended the species, describing the exo-erythrocytic parasite stages, as seen in T. parva infections. In addition, Dschunkowsky (1927, 1952) proposed a mode of intraerythrocytic schizogonous multiplication for T. annulata which resulted in the formation of 2-4 anaplasmoid daughter parasites.

The best supportive evidence for the distinction between T. parva and T. annulata was derived from the cross immunity trials conducted in Algeria by Sergent, Donatien, Parrot, Lestoquard and Plantureux (1927). These extensive studies were conducted cn Theileria dispar (Sergent et al., 1945) which was subsequently recognized as being synonymous with T. annulata (Dschunkowsky, 1948; Barnett, 1963).

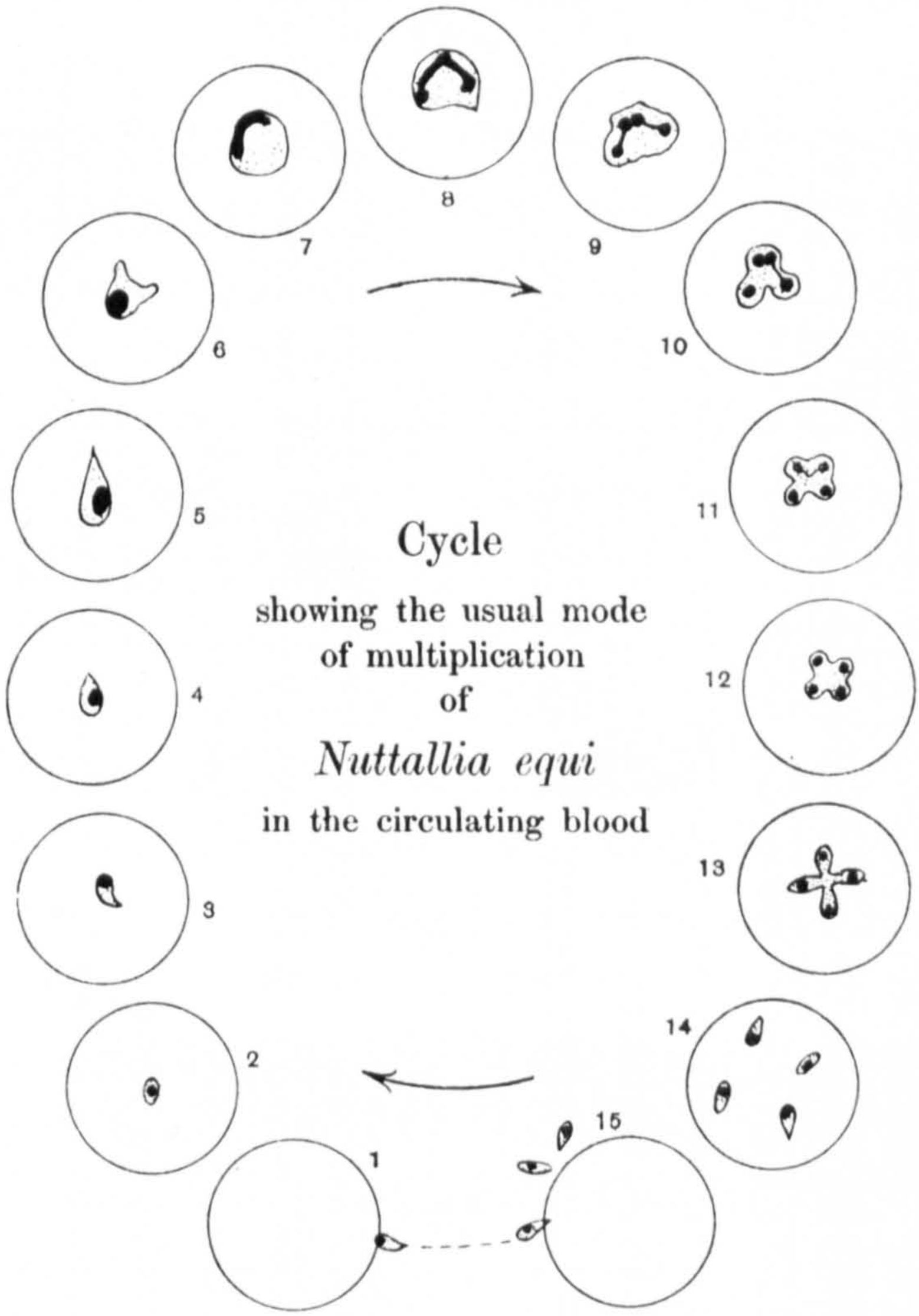
One important point, often ignored in the taxonomic discussion, is that the definitive characteristic of Theileria, as proposed by Bettencourt et al. (1907) was the division of the intraerythrocytic piroplasms into four small parasites arranged in a cross configuration. The cross forms which they found in the erythrocytes of the fallow deer were analogous to the anaplasmoid forms in tropical theileriosis (Dschunkowsky and Luhs, 1904) and identical to those described by Koch (1905) in cattle infected with T. parva (Bettencourt et al., 1907). Koch (1905) considered the intraerythrocytic division into four (⁘) as a major characteristic differentiating the etiologic agent of ECF from P. bigemina, and suggested that the piroplasms which divided into cross forms, including those seen in horses (Laveran, 1901) be grouped together taxonomically.

The small piroplasm of horses, which is now recognized as Babesia equi (Laveran, 1901), was named Nuttallia equi by França (1909). Nuttall and Strickland (1910, 1912) observed the division of N. equi into four daughter parasites within erythrocytes, in living and fixed preparations of blood from infected horses (Figure 1.2). Similar cross forms were seen in the erythrocytes of cattle infected with T. parva (Figure 1.3) but these parasites were never observed in the process of division (Nuttall, Fantham and Porter, 1909; Nuttall and Fantham, 1910). Nuttall and his associates concluded from their in vitro and in vivo studies that if T. parva multiplied within erythrocytes the process of division, into 2 or 4 parasites, was very slow.

The ability of T. annulata and T. mutans piroplasms to divide was generally accepted but the mode of multiplication has remained uncertain. Intraerythrocytic schizogony, as proposed for the two parasites respectively by Dschunkowsky (1927, 1952) and Oteng (1971) has not been widely accepted. Electron microscopic studies of blood from cattle infected with T. mutans (Büttner, 1966) and T. annulata (Schein, Mehlhorn and Warnecke, 1977) indicated that the piroplasms divided by binary fission. The proponents of intraerythrocytic binary fission (Neitz, 1959; Mehlhorn, 1982; Saidu, 1982) or a combination of binary and quadruple fission (Ishihara and Ishii, 1958; Levine, 1973; Soulsby, 1982) have tended to predominate.

Controversy prevails as to whether T. parva divides in the erythrocyte and if so, by what mode. Although early investigators believed that intraerythrocytic multiplication could occur in

Figure 1.2 Primary mode of multiplication for Nuttallia equi (synonym Babesia equi) proposed by Nuttall and Strickland (1912)



Cycle

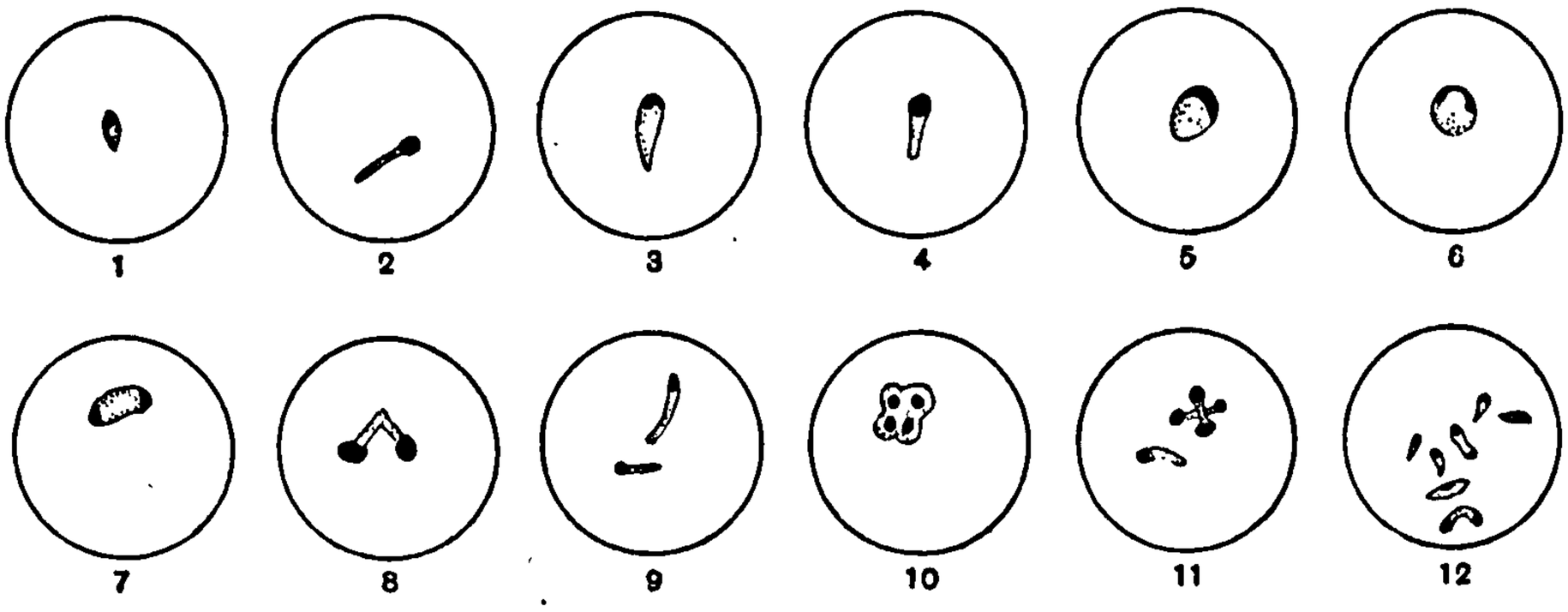
showing the usual mode
of multiplication
of

Nuttallia equi

in the circulating blood

Figure 1.3 Intraerythrocytic stages of Theileria parva diagrammatically illustrated by Nuttall and Fantham (1910).

After extensive observations Nuttall and Fantham (1910) concluded that T. parva "may divide into two or four parasites at a time, although division has not been observed to take place in living parasites".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T. parva infections (Koch, 1905; Bettencourt et al., 1907; Nuttall et al., 1909), Gonder did much to dispel this conviction. He proposed that intraerythrocytic stages of T. parva were gametocytes that completed their sexual development as microgametes and macrogametes in the engorged tick (Gonder, 1910, 1911a,b). Gonder admitted that differentiation between the cruciate configuration of the parasites in T. parva and those of the species which he called Babesia mutans was difficult. Gonder (1911b) contended, however, that "the cross shapes of Babesia mutans result directly from the macrogametes by means of parthenogenesis, whilst the quadruplet forms of Theileria parva are produced from gamonts by means of gamogony". According to Gonder's theory small portions of the intralymphocytic gamonts (microschizonts) occasionally invaded erythrocytes where they completed division.

The distinctly different interpretations given to strikingly similar developmental forms of B. mutans, B. equi and T. parva are illustrated in Figure 1.4 which was taken from a classic protozoology text (Wenyon, 1926, 1965).

Creative, but cautious, interpretations of the developmental stages of T. parva in cattle were presented after detailed studies by Cowdry and Danks (1933) who acknowledged the possible significance of the cross forms in intraerythrocytic multiplication. The authors suggested, however, that binary division should also be considered and that cross forms might be due to the close apposition of two dumb-bell shaped piroplasms.

The next major investigation of the developmental stages of T. parva in cattle and ticks was conducted by Reichenow (1940) who

Figure 1.4 Photographic reproduction of original drawings of Babesia and Theileria, as seen in Romanowsky stained blood-films and, accompanying descriptions from Wenyon (1926).

16-20 Babesia mutans (synonym Theileria mutans)

"It appears that reproduction is effected by division into two, of which the dumb-bell form is a stage, or by division into four resulting in the cross forms in which four minute pear-shaped individuals radiate from a central point."

(p. 1002)

26-30 Babesia equi

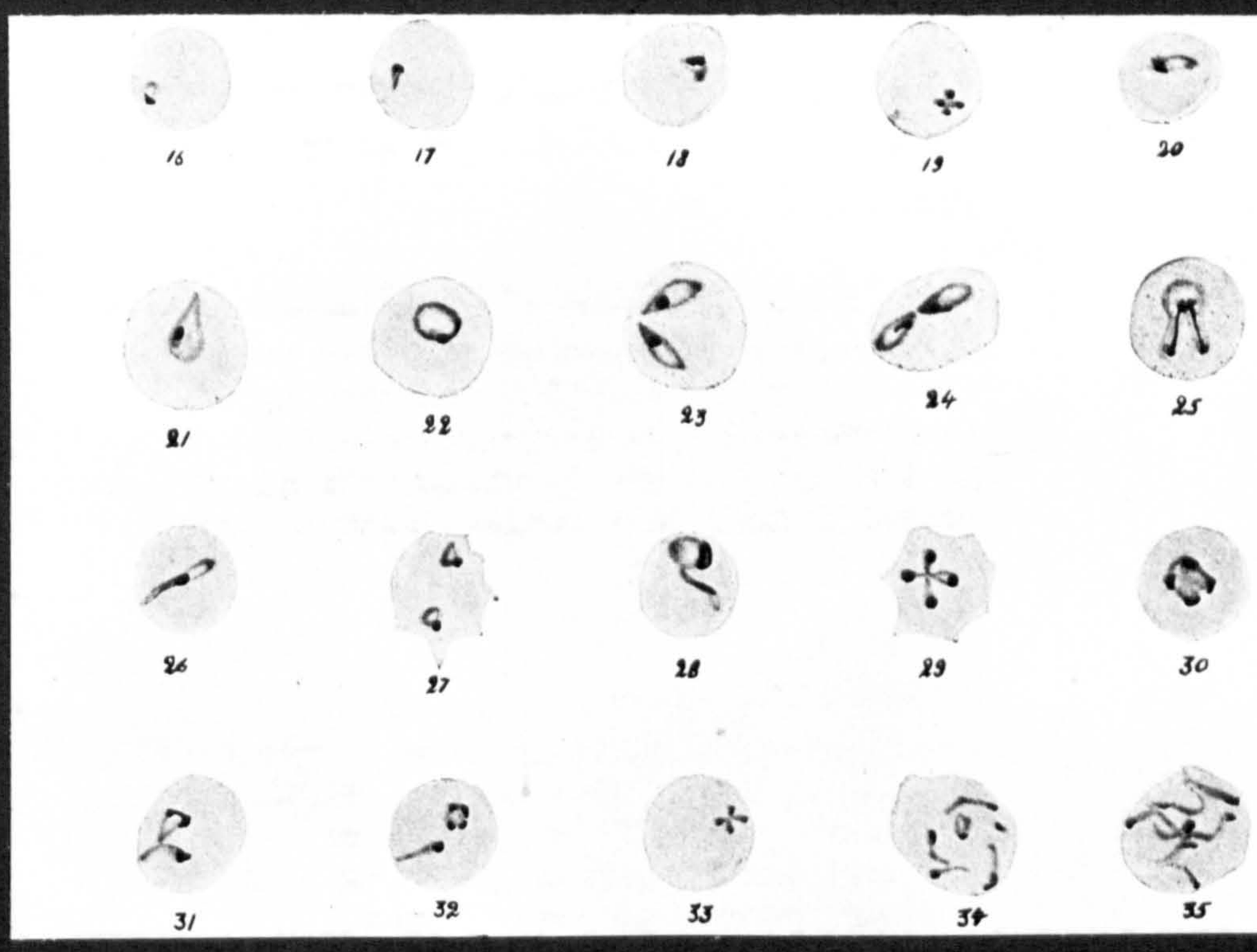
"The nucleus divides into two parts and then each of these divides again. Finally, the cytoplasm buds off four daughter forms which radiate from a central point, giving rise to the characteristic cross forms."

(p. 1010)

31-35 Theileria parva

"Though they may sometimes be seen in pairs in the red cells or occasionally in fours as in the cross forms, it is doubtful if these represent division stages, as they do in the case of B. mutans, the morphological resemblance to which is very striking."

(p. 1031)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concluded that the only phase of multiplication in the mammalian host was due to a process of multiple binary division in the lymphocytes. Interestingly, in the same paper, Reichenow applied his theory of binary division to explain the cross forms in the genus Nuttallia but failed to mention or illustrate similar forms in T. parva infections.

All of the aforementioned opinions had a profound effect on the study of Theileria but none were more influential than those presented by Neitz. After studying the life cycle of the bovine Theilerias in pure, tick transmitted infections, Neitz and Jansen (1956) concluded that a major revision of the classification system was justified. The primary basis for the revision was the absence of intraerythrocytic multiplication in the life cycle of T. parva. The supportive evidence proposed was the failure to consistently transmit ECF by the inoculation of infected blood (Theiler and du Toit, 1929; Neitz, 1948), to induce relapses by splenectomy (du Toit, 1931; Barnett and Bailey, 1955a) or to identify persistent carriers after recovery (Stockman, 1905; Neitz and Jansen, 1956), all of which were shown to be possible with the other bovine theilerial parasites (Theiler and Graf, 1928; du Toit, 1930; Adler and Ellenbogen, 1935; Sergent et al., 1931; Sergent et al., 1945; Neitz, 1956). In the new classification system T. parva was the only parasite retained in the family Theileriidae. The other parasites, including T. mutans, T. annulata and the recently recognized T. lawrencei (Neitz, 1955), which all divided into 2 or 4 in the erythrocytes, were placed in the new family Gonderiidae in the resurrected genus Gonderia (du Toit, 1919).

The concept of sterile immunity which was so fundamental to Neitz's arguments and to ECF control programmes, became suspect when Barnett (1956a) showed that after recovery from a T. parva infection some animals remained infective for ticks. Barnett (1956b) was also able to serially passage a Kenyan strain of T. parva by blood inoculation. The possibility existed, however, that macroschizont infected lymphoid cells persisting in the chronic carrier state and present in the blood inocula could have accounted for the observations made in these experiments. Only after Neitz (1964, 1965) conducted his own series of transmission experiments was he convinced that T. parva piroplasms could be maintained for periods up to three years, by intraerythrocytic multiplication in the absence of schizonts.

The genus Gonderia was abolished (Cheissin, 1965; Levine, 1971) but the question of piroplasm multiplication in ECF was not resolved (Barnett, 1968). Jarrett, Crighton and Pirie (1969) argued that if intraerythrocytic multiplication occurred in a T. parva infection then, in relation to the parasitaemia, the number of multi-parasitized erythrocytes should vary significantly from the number which could be predicted, by Poisson distribution, to occur from the random invasion of erythrocytes by merozoites. Sample counts made from the blood smears of different cattle with ECF, however, showed a close correlation between the calculated and observed values for the distribution of piroplasms.

Electron microscopic studies of the intraerythrocytic stages of T. parva, in bovine blood with 50-90% parasitaemia, failed to reveal ultrastructural features suggestive either of multiplication

or of sexual dimorphism (Büttner, 1967a).

The development of extracellular spindle-shaped forms with single spikes and several ray-like projections, propounded to be microgametes, were observed by light and electron microscopy in the gut of T. annulata-infected Hyalomma anatolicum excavatum (Schein, 1975; Schein et al., 1975; Mehlhorn, Weber, Schein and Büscher, 1975) and in T. parva-infected H. a. excavatum (Mehlhorn and Schein, 1976) and R. appendiculatus (Schein, Warnecke and Kirmse, 1977). The larger (3-4 μ) spherical forms of the parasites seen in the same ticks were considered to be macrogametes. The microgametes resembled the sexual stages of T. parva described by Koch (1905, 1906) who referred to these, and similar forms seen in P. bigemina-infected ticks, as "Strahlenkörper".

Although syngamy has not been observed, the probable sexual significance of parasitic forms with distinctive spiky projections or "Strahlen", and the ultrastructural features of an apical complex in the merozoites of Theileria, were considered amongst the taxonomic criteria for reclassifying the species with Babesia, in the phylum APICOMPLEXA (Levine et al., 1980).

Regarding intraerythrocytic multiplication, the prevailing opinion was expressed by Levine (1971) who stated that in Babesia the "trophozoites multiply by binary fission in the erythrocytes forming pairs or by schizogony forming tetrads" but in Theileria "forms in the erythrocytes may or may not reproduce".

1.3 In vitro Cultivation of Theileria and Babesia

The potential for *in vitro* cultivation of *Babesia* and *Theileria* intrigued even the earliest discoverers of the parasites. Babes (1890) was the first to claim the successful isolation of *B. bovis in vitro* but the organisms grown in his cultures were more likely to have been bacteria (Wenyon, 1926). A report by Lignières (1903) that marked multiplication of *B. bovis* occurred after 15 days in cultures which he successfully passaged twice were also viewed with some scepticism (Nuttall and Graham-Smith, 1908). Dschunkowsky and Luhs (1904) claimed to have observed significant intraerythrocytic multiplication of *T. annulata* after 10-15 days in cultures of defibrinated, infected blood. Although the parasites showed definite motility in the erythrocytes and serum for 20-25 days *in vitro* at room temperature or at 38°C, long term cultures were not established and the parasites lost their infectivity for cattle.

In an effort to study the "Strahlenkörper" of *T. parva* (Koch, 1905, 1906), Miyajima (1907) established cultures with defibrinated blood from Japanese cattle and grew large, flagellate parasites which he believed to be developmental stages of *T. parva*. Miyajima was probably attempting to culture *T. sergenti/orientalis*, since *T. parva* does not occur in Japan (Minami, Ishihara, and Fujita, 1981; Uilenberg, 1981) and was successfully growing trypanosomes, perhaps *Trypanosoma theileri*. Nuttall and Graham-Smith (1909) were unable to reproduce Miyajima's results in Britain using blood from cattle that were infected with *T. parva* from *Rhipicephalus evertsi* ticks, imported from South Africa.

Kleine (1906) and Nuttall and Graham-Smith (1908) had more success, however, with cultures of Babesia canis. "Strahlenkörper" were observed in the upper erythrocyte layer of B. canis-infected blood which had been mixed in equal volumes with either physiological saline or simple salt solutions and incubated for 24-72 hours at 24-27°C. At temperatures above 32°C B. canis was observed, for brief periods, to divide by a budding process in the erythrocyte, escape, and invade other erythrocytes (Nuttall, 1908). Nuttall and Graham-Smith (1908) reviewed the early efforts of others to culture Babesia ovis, B. equi, B. bovis and Theileria spp.

Bass and Johns (1912) were the first to maintain the intraerythrocytic stages of the malarial parasites Plasmodium vivax and P. falciparum through several generations of schizogony in stationary cultures of defibrinated blood mixed with 50% dextrose solution. Modifications of the Bass and Johns' method were used to cultivate B. canis (Knuth and Richters, 1913; Thomson and Fantham, 1914; Ziemann, 1913). In Holland, Vrijburg (1913) was unsuccessful in applying this method to the cultivation of a parasite that he believed was a small variety of Babesia bigemina, similar to the parasite described in North Africa and Germany but distinct from Babesia divergens.

Little significant progress was made in the following 50 years towards developing long-term continuous cultivation systems for intraerythrocytic protozoal parasites. Successful cultures of the exoerythrocytic stages of avian malarial parasites did, however, provide valuable information which was subsequently used by tissue culturists working with mammalian malarial parasites and Theileria.

Encouraged by the early endeavours of Huff and Bloom (1935) to grow Plasmodium elongatum in bone marrow cells from infected canaries, Gavrilova, Bobkoff and Laurencin (1938) utilized plasma clot and hanging drop techniques to maintain Plasmodium gallinaceum in bone marrow explants (cited by Hawking, 1944). Subsequently, a technique was developed whereby sporozoites of P. gallinaceum, from salivary glands of Aedes aegypti mosquitoes, placed in cultures of normal macrophages from the spleen of chick embryos, invaded the cells and transformed into cryptozoites (Dubin, Laird and Drinnon, 1949, 1950). Reviews by Hawking (1951), Meyer and Musacchio (1959), Huff (1964) and Trager and Jensen (1980) describe the methods by which the exoerythrocytic stages of various avian malarial parasites have been successfully cultivated for over 40 years.

The methods presently used for cultivating the exoerythrocytic, macroschizont stages of Theileria in lymphoid cells evolved from those initially employed in Israel by Tsur-Tchernomoretz (1945) to maintain T. annulata in plasma clot cultures of infected lymph node and spleen tissue. The presence of normal bovine or rodent spleen explants in the cultures appeared to enhance parasite survival and long-term cultures were eventually maintained for months with serial subcultures (Tsur-Tchernomoretz, 1947; Tsur-Tchernomoretz and Pipano, 1959). Cultures were later established in flasks using infected bovine tissues which were trypsinized and resuspended in medium consisting of Earle's solution, bovine serum, yeast extract and antibiotics (Tsur and Adler, 1963). After the centrifugation of blood from cattle with tropical theileriosis, chicken embryo extract was placed on the buffy coat layer to enhance coagulation, the clotted

leucocytes removed, trypsinized and subsequently used to initiate similar cultures (Tsur and Adler, 1965).

In cultures of T. annulata-infected tissues, the parasitized lymphoid cells generally grew in association with the fibroblastoid cell monolayer which was formed naturally by adherent cells. Hooshmand-Rad and Hashemi-Fesharki (1968) in Iran observed that once a significant number of infected lymphoid cells were present in the supernatant medium of T. annulata tissue cultures, aliquots of these cell suspensions could be passaged and new cultures established without monolayers. Although the cultures were essentially stationary, the authors called them "suspension cultures" to emphasize that the growth of large numbers of T. annulata-infected lymphoid cells did not require monolayers or the presence of any other cells (Hooshmand-Rad and Hashemi-Fesharki, 1968; Hooshmand-Rad, 1975).

The major incentive for growing T. annulata in vitro was the potential utilization of these exoerythrocytic stages in a vaccine. Cattle could be premunized by the inoculation of bovine blood or other tissues containing macroschizont infected lymphoid cells (Sergent et al., 1945). The ensuing reactions were usually less severe than those induced by tick-transmitted infections. However, significant mortalities could result from the artificial inoculation of virulent parasite strains, and nonvirulent strains did not always provide protection against the more pathogenic strains of T. annulata. Fortunately, the prolonged maintenance in vitro of T. annulata-infected lymphoid cells proved effective in attenuating the virulence, without decreasing the immunogenicity of the parasite, and provided the basis for a vaccine (Tsur, Adler, Pipano and Senft, 1964; Tsur,

1965; Pipano and Tsur, 1966; Pipano, Klopfer and Cohen, 1973).

Attempts during the same period to isolate a mild strain of T. parva for use in a vaccine or to attenuate the highly pathogenic parasite by serial mechanical passage through cattle, cyclical transmission through abnormal vectors or inoculation into laboratory animals were uniformly unsuccessful (Barnett and Bailey, 1955b; Barnett, 1956c). The potential practical applications of a tissue culture system for T. parva prompted the East African Veterinary Research Organization to recruit a veterinary parasitologist with experience in culture techniques for avian malarial parasites. Using primarily infected lymph node and splenic tissue, Brocklesby (1956) attempted, without success, to grow T. parva in trypsinized monolayers, in biopsies implanted on coverslips and incubated in candle jars, and in the plasma clot cultures described by Tsur-Tchernomoretz (1945).

Similarly, Tsur was only able to maintain T. parva infected lymphocytes for 15 days in vitro with a cessation of parasite multiplication observed during the first week (Tsur, Neitz and Pols, 1957). The behaviour and fastidious requirements of T. parva in vitro were well demonstrated in a series of experiments by Brocklesby and Hawking (1958) who managed to successfully establish cultures of T. annulata but could not maintain T. parva for longer than 14 days.

After these frustrating failures the announcement by Hulliger and her colleagues that long-term cultures of T. annulata, T. parva, and subsequently T. lawrencei had been established in their laboratory seemed to be a breakthrough (Hulliger, Wilde, Brown and Turner,

1964; Hulliger, 1965). However, cross immunity trials later indicated that all of the cultures were immunologically identical to the Tova strain of T. annulata (Wilde, 1967). The possibility existed that due to cross contamination, T. annulata-infected lymphocytes had proliferated in all of Hulliger's original cultures.

Credit for the establishment of the first T. parva-infected lymphoid cell lines is given therefore, to Malmquist, Nyindo and Brown (1970), who worked exclusively with this parasite (Brown, 1979a). The purity of Malmquist's cell lines was confirmed by the inoculation of cattle with culture material (Brown, Malmquist, Cunningham, Radley and Burridge, 1971). The mammalian phase of the parasite's life cycle was completed and adult ticks, that fed as nymphs on the infected animals, subsequently induced a classical ECF reaction when placed on susceptible cattle. Hope of a tissue culture vaccine was revived when the majority of cattle receiving between 10^5 and 10^9 culture-derived T. parva-infected lymphoblasts suffered mild or inapparent reactions, and resisted homologous challenge with sporozoites (Brown et al., 1971; Brown, Crawford, Kanhai, Njuguna and Stagg, 1978).

The astute observations of Hulliger on the behaviour of Theileria in infected lymphoblastoid cell cultures were nonetheless significant. She concluded from her studies that the division of the macroschizont and host cell were interdependent and that in vitro Theileria was maintained by the clonal proliferation of the parasitized lymphoid cells (Hulliger et al., 1964; Hulliger, 1965). This thesis refuted the prevailing theories that intralymphocytic schizonts of Theileria produced merozoites which then invaded other lymphocytes to maintain

the parasitic cycle in cattle (Gonder, 1910, 1911a,b; Reichenow, 1940; Cowdry and Danks, 1933).

The basic mode of exoerythrocytic multiplication for Theileria species proposed by Hulliger was confirmed by subsequent in vitro and in vivo studies (Jarrett, Crighton and Pirie, 1967; Moulton Krauss and Malmquist, 1971; De Martini and Moulton, 1973; Radley, Brown, Burridge, Cunningham, Peirce and Purnell, 1974; Musisi, Bird, Brown and Smith, 1981; Irvin, Ocama and Spooner, 1982). A behavioural characteristic, fundamental to the propagation and pathogenicity of the parasite, was revealed in a culture system after eluding investigators for over 60 years.

Improved culture techniques, as reviewed by Brown (1979a, 1980), now facilitate the establishment of Theileria cultures from a variety of tissues, as well as from blood taken from infected cattle. A significant milestone was passed when a method was developed by which T. parva sporozoites, obtained from infected R. appendiculatus either by grinding the partially fed ticks (Purnell, Brown, Cunningham, Burridge, Kirimi and Ledger, 1973) or artificially inducing them to salivate (Purnell and Joyner, 1967; Cunningham, Brown, Burridge, Joyner and Purnell, 1973), were used to infect monolayer cultures of normal bovine lymphoid cells and establish Theileria cell lines (Brown, Stagg, Purnell, Kanhai and Payne, 1973; Brown, 1979b).

The host-parasite relationship at the site of tick attachment and in the lymphatic system are difficult to investigate in vivo. The in vitro infection technique has been used to study the invasive behaviour and early developmental stages of T. parva (Kurtti,

Munderloh, Irvin and Büscher, 1981; Stagg, Dolan, Leitch and Young, 1981) and T. annulata (Jura, Brown and Kelly, 1983). Theories concerning the effect of humoral factors on the invasion of sporozoites and the preference of Theileria for lymphocytes of different types or species of Bovidae were tested with this system (Spooner and Brown, 1980; Gray and Brown, 1981; Dolan, Njuguna and Stagg, 1982; Stagg, Young, Leitch, Grootenhuis and Dolan, 1983; Musiime, 1983).

The use of culture-derived Theileria-infected lymphoid cells as the basis for vaccines, has been reviewed by Pipano (1980, 1981) and Brown (1981). Infected lymphoblasts from cultures are commonly used as the macroschizont antigen in the indirect fluorescent antibody test (Burridge and Kimber, 1972; Goddeeris, Katende, Irvin and Chumo, 1982) and in the electrophoretic separation of isoenzymes (Melrose, Brown and Sharma, 1980; Musisi, Kilgour, Brown and Morzaria, 1981). Both of these techniques have been used to differentiate Theileria species. Valuable information has been acquired from immunological studies which made use of autologous and allogeneic infected cell lines to ascertain the significance of genetic restriction in the cell mediated immune response to T. parva (Pearson, Lundin, Dolan and Stagg, 1979; Emery, Morrison, Nelson and Murray, 1981a; Emery, Morrison, Büscher and Nelson, 1982; Pearson, Hewett, Roelants, Stagg and Dolan, 1982; Emery and Kar, 1983) and T. annulata (Preston, Brown and Spooner, 1983). Drugs effective against theilerial macroschizonts were also produced after the efficacy of compounds was tested in Theileria-infected lymphoid cell cultures (McHardy, 1978; Morgan and McHardy, 1982). There appear to be innumerable potential

applications for the in vitro cultivation techniques in theilerial research.

The present systems are, however, limited to the cultivation of only one part of the mammalian life cycle of Theileria. Microschizonts are occasionally observed in established T. parva cultures (Brown, 1979a; Kurtti et al., 1981) and rarely, if ever, seen in cultures of T. annulata (Pipano, 1977; Shad-del, 1977). The appearance of microschizonts in cultures incubated at elevated temperatures of 41-42°C reported by Hulliger et al., (1966) is remarkable, therefore, if the cultures were infected with T. annulata rather than T. parva.

Increased levels of foetal bovine serum and the addition of 5-10% bovine lymph to T. parva cultures appeared to stimulate the formation of microschizonts and the production of merozoites which were capable of invading normal bovine erythrocytes added to the cultures (Danskin and Wilde, 1976a, b). Nyindo and co-workers reported having seen, by electron microscopy, piroplasms which resulted from the in vitro invasion of exoerythrocytic merozoites, but they were unable to identify similar forms in culture samples using light microscopy (Nyindo, Kaminjolo, Wagner and Lule, 1978). The same results using methods described in the aforementioned papers for the induction of merogony, resulting in merozoites which invade erythrocytes in vitro, have not been reproduced by other investigators (Shad-del, 1977; Uilenberg and Pipano, 1981).

Completion of the theilerial life cycle is dependent upon the infection of larvae or nymphs of the vector ticks by the

intraerythrocytic stages of the parasite. Nymphal ticks can be artificially infected by feeding on capillary tubes containing parasitaemic blood (Purnell, Branagan, Bailey, Joyner and Radley, 1970) or on rabbits inoculated with theilerial piroplasms (Purnell, Irvin, Kimber, Omwoyo and Payne, 1974; Irvin, Purnell, Brown, Cunningham, Ledger and Payne, 1974). Parasitized blood can also be injected percutaneously to infect ticks directly (Schreuder and Uilenberg, 1976; Walker, Brown, Bell and McKellar, 1979). An attempt to infect ticks by the percutaneous inoculation of concentrated T. parva-infected lymphoid cell cultures containing microschizonts and extracellular merozoites was, however, unsuccessful (Jongejan et al., 1980). Therefore, until the mammalian stages of the life cycle of Theileria are completed in vitro, the piroplasms required to maintain colonies of infected ticks for the production of sporozoites must be obtained by the expensive procedure of infecting cattle.

In contrast the developmental cycle of Babesia equi, which normally occurs in horses, has apparently been completed in vitro (Schein, Rehbein, Voigt and Zweygarth, 1981). The techniques used to prepare B. equi sporozoite suspensions from H. a. excavatum ticks, to infect lymphocytes isolated from the peripheral blood of uninfected horses in vitro and subsequently establish parasitized lymphoblastoid cell lines, are essentially the same as those used for Theileria (Rehbein, Zweygarth, Voigt and Schein, 1982). The appearance and behaviour of the intralymphocytic stages of B. equi, including the synchronous division of the macroschizont and host cell, bear a striking resemblance to those of Theileria (Schein et al., 1981;

Moltmann, Mehlhorn, Schein, Rehbein, Voigt and Zweygarth, 1983a). Microschizonts appeared nine days after the *in vitro* sporozoite infection, producing merozoites which invaded normal equine erythrocytes and subsequently divided into "maltese" cross forms (Schein *et al.*, 1981).

The major breakthrough in the cultivation of intraerythrocytic protozoan parasites has undoubtedly been the establishment by Trager and Jensen (1976) of long term cultures of *Plasmodium falciparum*. After decades of work trying to culture various avian and mammalian species of *Plasmodium* (Trager, 1941, 1943, 1950) these scientists developed two techniques for the *in vitro* maintenance of the most pathogenic of the human malarial parasites. The two methods employed are similar in concept and have served as the basic model used in the development of cultivation systems for other species of *Plasmodium* (Chowduri, Chowdhury and Regis, 1979; Wickham, Dennis and Mitchell, 1980; Zhengren, Minxin, Yuhua, Shumin and Nailin, 1980; reviewed by Trager, 1982; Guo, Chin and Collins, 1983) and for *Babesia* (Erp, *et al.*, 1978; Väyrynen and Tuomi, 1982; G. Konrad, personal communication).

In both the continuous flow-vial and the candle jar methods used by Trager and Jensen (1976) the *P. falciparum*-infected blood was mixed with uninfected human erythrocytes to reduce the parasitaemia below 2%, and a shallow settled layer of erythrocytes was maintained with no more than 2-3 mm of overlying medium. Cultures in the automated flow-vial system received a continuous flow of fresh medium and a gas mixture of 1-5% oxygen (O_2) and 7% carbon dioxide (CO_2) in nitrogen. The candle jar method was simpler in

that the atmosphere of relatively high CO_2 (3%) and reduced O_2 (approximately 17%) was created by burning a candle in a closed glass desiccator jar containing the cultures. In both systems at least a six-fold increase in the parasitaemia was observed every 48 hours.

Modifications were subsequently made to the P. falciparum culture systems for convenience (Jensen and Trager, 1977, 1978; Trager, 1979; Trager and Jensen, 1978, 1980), to increase the production of merozoites (Chin, 1979; Butcher, 1981; Siddiqui and Palmer, 1981; Zolg, McLeod, Dickinson and Scaife, 1982; Palmer, Hui, Siddiqui and Palmer, 1982) and to promote gametocytogenesis (Carter and Beach, 1977; Sinden and Smalley, 1979; Brockelman, 1982; Campbell, Collins, Nguyen-Dinh, Barber and Broderson, 1982; Ponnudurai, Meuwissen, Leeuwenberg, Verhave and Lensen, 1982; Sinden, 1983).

While malariologists struggled with the initial problems of cultivating the intraerythrocytic stages of Plasmodium, little progress was made with Babesia beyond the growth in short-term cultures. Chiodini (1973a) reported multiplication in cultures of Babesia divergens, B. canis and B. rodhaini over 34-48 hours in vitro and used these cultures to study the effect of babesiacides on the parasites (Chiodini, 1973b). Wyss (1976) tried various stationary and suspension culture systems but was unable to maintain the growth of Babesia bigemina in vitro. Although the parasitaemia of B. microti has been reported to double or triple within 24-48 hours in vitro, using the candle jar technique (Bautista and Kreier, 1979, 1980; Coombs and McGill, 1980), growth apparently

could not be maintained beyond 96 hours. Timms (1980) observed multiplication of B. rodhaini, B. bovis and B. bigemina in short-term cultures, but only in the B. bigemina cultures did the parasitaemia rise above 1% before degeneration began after 24-48 hours in vitro.

The candle jar method was used with limited success by Erp et al. (1978) to cultivate B. bovis, whereas in parallel cultures, where erythrocytes were suspended by slow stirring in spinner flasks, significant multiplication was observed with maximum parasitaemias attained of 10-15%. The use of defibrination rather than chemical anticcagulants for the blood, a complex tissue culture medium with 40% normal bovine serum, and a gaseous environment of 5% CO_2 in air were amongst the propitious factors identified for the cultivation of B. bovis (Erp, Smith, Ristic and Osorno, 1980a).

B. bovis was continuously propagated in the improved suspension system for prolonged periods without any observable change in morphology or infectivity for cattle (Erp, Smith, Ristic and Osorno, 1980b). Preliminary vaccination trials suggested that a culture derived soluble antigen combined with saponin adjuvant could induce a protective immune response in cattle against B. bovis (Smith, Carpenter, Cabrera, Gravely , Erp, Osorno and Ristic, 1979; Smith, James and Ristic, 1981).

The prospect of a culture-derived vaccine inspired Levy and Ristic (1980) to develop a stationary erythrocyte culture system which allowed for a faster rate of in vitro multiplication and consistently higher B. bovis parasitaemias. An erythrocyte concentration of 5-10% (v/v), and a total depth of 0.62 cm for the

medium and settled erythrocyte layer, were considered critical in their microaerophilous stationary phase (MASP) culture system. The overlying medium acted as an oxygen barrier and Levy and Ristic proposed that the microaerophilic environment was created as the oxygen in the erythrocyte layer was consumed by the metabolizing parasites. The focus of attention in relation to B. bovis cultures is currently on the isolation of non-viable soluble antigens (James, Levy and Ristic, 1981; James, Kuttler, Levy and Ristic, 1981) and their efficacy as a vaccine compared to the live, attenuated B. bovis vaccines currently produced (Kuttler, Levy, James and Ristic, 1982; Timms, Dalgleish, Barry, Dimmock and Rodwell, 1983).

The MASP system has also been used to cultivate B. canis for the production of soluble antigens (Molinar, James, Kakoma, Holland and Ristic, 1982; Laurent, Moreau, Levy and Ristic, 1982). Parasitaemias of 10-15% are obtained in vitro but continuous MASP cultures of B. canis have not yet been established (N. Laurent, personal communication).

Väyrynen and Tuomi (1982) established continuous cultures of B. divergens using the medium and erythrocyte concentrations recommended for the MASP system while incubating the cultures in candle jars. B. divergens has been successfully maintained in modified stationary culture systems with reduced concentrations of bovine erythrocytes (G. Konrad, personal communication) and with human erythrocytes (M. Pudney, personal communication).

Advances made in the cultivation of the intraerythrocytic stages of Plasmodium and Babesia stimulated interest in the prospect of applying similar methods to propagate the piroplasms of Theileria

in vitro. A continuous cultivation system for the intraerythrocytic theilerial parasites would serve as a valuable source of experimental material and perhaps, another step towards the completion *in vitro* of the parasite's life cycle.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Protozoal Parasites

2.1.1 Theileria: Two strains of Theileria annulata and a single strain of Theileria parva, referred to by the location of the original parasite isolation, were used in experiments. The Hissar strain of T. annulata was isolated in India (Gill, Kaur and Bhattacharyulu, 1974) and made available to the Centre for Tropical Veterinary Medicine (CTVM) in 1978 by Professor B.S. Gill and Dr. Y. Bhattacharyulu, Ludhiana, India, in a batch of infected Hyalomma anatolicum anatolicum (Ludhiana) ticks. The Ankara strain of T. annulata originated from Turkey (Schein et al., 1975) and was supplied in infected Hyalomma anatolicum excavatum ticks by Professor E. Schein, Berlin, West Germany, in 1977. The Muguga strain of T. parva was isolated by staff members of the East African Veterinary Research Organisation in Kenya (Brocklesby, Barnett and Scott, 1961) and brought to the CTVM in infected Rhipicephalus appendiculatus (Muguga) ticks in 1977.

The ticks were maintained in colonies at the CTVM by standard techniques (Bailey, 1960; Irvin and Brocklesby, 1970; Hosie and Walker, 1979; Walker, Fletcher, McKellar, Bell and Brown, 1983). H. a. anatolicum and R. appendiculatus nymphs were infected by feeding on calves exhibiting, respectively, T. annulata and T. parva piroplasm parasitaemias. The H. a. anatolicum ticks were originally supplied by Professor B.S. Gill and Dr. Y. Bhattacharyulu of Ludhiana, India. The R. appendiculatus ticks used were derived from a batch of ticks specially selected from R. appendiculatus (Muguga) for

increased susceptibility to T. parva infection by Professor E. Schein, Berlin, West Germany.

2.1.2 Babesia: Two isolates of Babesia bovis, designated as the Mexican and South African strains, were provided by Dr. N. McHardy of Wellcome Research Laboratories, Beckenham, England, in samples of infected bovine blood. The Mexican isolate (Smith, Osorno, Brener, De La Rosa and Ristic, 1978) had been established in vitro from the blood of an infected calf in Mexico (Erp et al., 1978) and maintained in cultures at the University of Illinois, Urbana, Illinois, U.S.A. (Levy and Ristic, 1980). Cultures were provided by Professor M. Ristic to Wellcome Research Laboratories, Beckenham, Kent and subsequently used to infect a splenectomized calf. Defibrinated blood, with a parasitaemia of less than 0.1%, was collected from the infected calf, sent to the CTVM and used to establish continuous B. bovis cultures on 22.7.82. The South African isolate was originally obtained from a splenectomized calf infected with the Onderstepoort vaccine strain of B. bovis (Taylor and McHardy, 1979) at the Kwanyanga Laboratory of Coopers (S.A.), East London, Republic of South Africa. Ten ml of heparinised blood from the calf, with a parasitaemia of 4%, was cryopreserved in glycerol and shipped as a stabilate by Dr. P.K. McKenzie to the Wellcome Research Laboratories. The original stabilate (EX 311) was subsequently sent to the CTVM on 14.3.83.

2.2 Cattle

Adult Bos taurus over 2 years of age used as normal blood donors were Jersey steers 45 and 46, Ayrshire steers 35 and 219, and Ayrshire cows 34 and 220. Ayrshire and Ayrshire/Shorthorn crossbred calves two to nine months of age, weighing 60-110 kg, were used for parasite transmission experiments.

2.3 Surgical Splenectomy of Cattle

Calves to be splenectomized were anaesthetized by the intravenous injection of 3 mg/kg of Saffan (Glaxo Lab.) intubated and maintained with 1.5-2.0% halothane gas anaesthetic (Fluothane, Imperial Chemical Industries).

An area on the left side of the body from the 11th rib to the point of the ilium and from the transverse processes of the lumbar vertebrae to a line parallel with the costochondral junctions of the caudal ribs was clipped, shaved, surgically scrubbed and rinsed with 70% ethanol. A 20 cm long skin incision was made beginning on a line parallel with the point of the ilium, approximately 2 cm caudal to the 13th rib and extending ventral, parallel to the rib. The external abdominal oblique, internal abdominal oblique, transverse abdominus and peritoneum were incised. The spleen was exteriorized and a double ligature of No. 1 chromic cat gut placed around the splenic artery and vein. After the vessels were incised, the spleen was removed. The peritoneum and abdominal musculature were closed with No. 1 chromic cat gut and the skin was closed with simple sutures using No. 2 nylon.

2.4 Preparation of Theileria Sporozoite Suspensions and Stabilates

Nymphal H. a. anatolicum and R. appendiculatus fed during a rising parasitaemia on calves infected with T. annulata or T. parva, respectively, were allowed to moult and harden during a 28 day incubation period at 28°C and 85% relative humidity. Adult ticks were partially fed on the ears of rabbits to stimulate theilerial sporoblast maturation. H. a. anatolicum was fed for three days and R. appendiculatus for four days.

The partially fed adult ticks were surface sterilized by washing once in 1% benzalkonium chloride (Roccal, Winthrop Lab.) three times in 70% ethanol and four times in Eagles Minimum Essential medium with Hanks salts (MEM-H) (Gibco Europe) supplemented with 200 iu benzylpenicillin (Crystapen, Glaxo Lab.), 200 µg streptomycin sulphate (Evans Medical) and 100 iu mycostatin (Nystatin, E.R. Squibb and Sons) per ml. After incubation in the final wash for 10 minutes, the ticks were transferred to a sterile mortar for grinding in MEM-H with 3.5% bovine plasma albumin (Armour Fraction V, Sigma Chemical) supplemented with 200 iu benzylpenicillin, 200µg streptomycin sulphate and 100 iu mycostatin per ml (MEM/BPA). The ticks were ground repeatedly in MEM/BPA and the supernate from each grind was collected. The total volume of MEM/BPA added was calculated so that the final pooled supernates contained four tick equivalents (t.e.) per ml. The final supernatant sporozoite suspension was centrifuged at 100 x g for five minutes (15°C) and the supernate retained. The pellet was resuspended in 3 ml MEM/BPA and reground in a Griffiths tube (Jencons Scientific). The two supernates were pooled and the final sporozoite suspensions filtered through an AP

prefilter (Whatman) and 8μ MF filter (Millipore).

Stabilates were prepared by mixing equal volumes of filtered sporozoite suspension and 15% (v/v) glycerol (Analar grade, BDH Chemicals) in MEM/BPA, dispensed into 2 ml screw-top glass vials (Wheaton Scientific) and placed at -70°C overnight. Stabilates were stored below -120°C in the gas phase of a LR40 liquid nitrogen container (Union Carbide, U.K.).

2.5 Preparation of Culture-derived B. bovis (Mexico) Stabilate

Fifty ml of a 10% erythrocyte suspension from cultures with a *B. bovis* (Mexico) parasitaemia of 9-10% were centrifuged at 3000 x g for ten minutes (5°C). The packed cells were resuspended in 1.5 ml of Puck's Saline G (Gibco Europe) supplemented with 20 g/L of D-glucose (BDH Chemicals) (PSG^+). An equal volume of 20% (w/v) polyvinylpyrrolidone (Pharmaceutical Grade, Sigma Chemical) in PSG^+ was added. The mixture was dispensed in 1.5 ml aliquots into screw-top glass vials and placed below -120°C in the gas phase of a LR40 liquid nitrogen container.

2.6 Infection of Cattle with Theileria and Babesia

2.6.1 Parasite inocula: The cattle infected for each experiment are listed in Table 2.1.

Theileria stabilates were thawed rapidly in a 37°C water bath and allowed to equilibrate at room temperature for 15-45 minutes prior to the inoculation of cattle. Calves received 1 ml of stabilate, containing 2 t.e., either in the shoulder area dorsal to the prescapular lymph node for *T. annulata* infections, or just below the left

Table 2.1 Cattle infected with Theileria and Babesia

Chapter*	Calf Number	Parasite Inoculum
3	117 118	T. annulata (Ankara) - sporozoite stabilate
3	121 122	T. annulata (Hissar) - sporozoite stabilate
3	127 128	T. annulata (Hissar) - fresh sporozoite suspension
3	145 146	T. annulata (Hissar) - sporozoite stabilate
3 3,5	156 153	T. annulata (Ankara) - sporozoite stabilate
4	194 195	T. annulata (Ankara) - sporozoite stabilate
5	148	T. annulata (Hissar) - sporozoite stabilate
6	154 155	T. annulata (Ankara) - sporozoite stabilate as primary and challenge infections
6	163 164	Filtered blood from calf 155 Unfiltered blood from calf 155
7	170 171	T. parva (Muguga) - sporozoite stabilate
8	198 199	B. bovis (South Africa) - blood stabilate B. bovis (Mexico) - culture-derived stabilate

*Parasitized blood or tissue samples from the calves listed were used in experiments described in the specified chapter of the thesis.

ear dorsal to the parotid lymph node for T. parva (see Table 2.1).

T. annulata (Hissar) sporozoite suspensions, containing either 1.0 or 0.1 t.e. were inoculated immediately after preparation into the subcutaneous tissue above the prescapular lymph node of calves 127 and 128, respectively. Calves 163 and 164 received intravenous and subcutaneous inoculations of defibrinated blood from a splenectomized T. annulata (Ankara) chronic carrier, calf 155.

Calf 198 received 4 ml of stabilate EX 311 which contained heparinized bovine blood infected with B. bovis (South Africa). Calf 199 was infected with 2 ml of a culture derived B. bovis (Mexico) stabilate, prepared as described in Section 2.5. Both stabilates were thawed rapidly in a 37°C water bath before inoculating half of the specified volume of parasitised material intravenously and half subcutaneously above the right prescapular lymph node.

2.6.2 Monitoring infections:

(a) Routine procedures - Temperatures were taken daily. Jugular blood was collected from cattle before infection and thereafter each week at least once for serum and three times for haematology. Leucocytes and erythrocytes were counted with a ZB1 electronic particle counter (Coulter Electronics), blood smears prepared and packed cell volumes determined (Schalm, Jain and Carroll, 1975) from each sample collected in a 5 ml vacutainer tube with 7.5 mg disodium edetate (Becton-Dickinson, U.K.). Serum samples were removed from clotted blood which was collected in 10 ml sterile vacutainers without additives (Becton-Dickinson, U.K.). The serum was stored at -20°C. Biopsy samples were taken from the right (RPG)

and left (LPG) prescapular lymph nodes of T. annulata-infected cattle with a 20 gauge needle. Smears prepared with blood and biopsy samples were stained and examined for parasites as described in Section 2.12.1.

(b) Serology: Indirect fluorescent antibody test - Macro-schizont antigen smears were prepared by a modification (M. Gray, personal communication) of the Burridge and Kimber (1972) technique using culture-derived T. annulata (Ankara) infected lymphoid cells. Fifty ml of culture suspension, with 10^6 lymphoid cells per ml, were centrifuged at 200 x g for ten minutes and the sedimented cells washed three times with PBS. The final cell pellet was resuspended in an equal volume of 1% (w/v) bovine plasma albumin (Fraction V, Sigma Chemical) in phosphate buffered saline (PBS - Dulbecco A, Oxoid) smeared on glass slides and fixed for ten minutes in acetone (Analar grade, BDH Chemicals). Smears were air dried and stored at -20°C in self-sealing polythene bags with silica gel.

The piroplasm antigen smears were prepared by M. Gray with the blood of calf 13 infected with T. annulata (Ankara) and stored at -20°C as above.

Pretested positive control serum from a calf infected with T. annulata (Ankara) was used at a 1:10 dilution in PBS. Autologous preinfection serum diluted 1:10 and PBS were the negative controls.

The test sera were diluted 1:10 with PBS and then by two-fold dilutions to 1:5120. One drop of each dilution of test serum and the controls were placed individually on circumscribed areas on the antigen smears. The smears were incubated for 30 minutes at room temperature in a humid chamber and then washed in three to four

changes of PBS. Aliquots of a 1:80 dilution of rabbit anti-bovine IgG globulin conjugated with fluorescein isothiocyanate (Nordic Immunological Lab.) in PBS were deposited on each test area. The smears were incubated as above for 30 minutes, washed in three to four changes of PBS, and drained.

A 1:10 dilution of Eriochrome Black A counterstain in PBS was applied for five minutes (Jones and Conrad, 1983). The smears were rinsed with PBS, air dried, and mounted with 24 x 64 mm glass coverslips. The mounting fluid was a 1:1 solution of PBS and glycerol (Difco Lab.) buffered with Tris (pH 9.7).

Smears were examined with the 54 x oel objective (0.94NA) on a fluorescence microscope (Orthoplan, Ernst Leitz Wetzlar) fitted with the vertical illuminator according to Ploem and a 200-watt ultra high pressure mercury vapour lamp. The BG38 and GG475 primary filters and a K530 suppression filter were used with a KP500 interference filter for selective fluorescein isothiocyanate excitement.

2.7 Culture Materials

Materials utilized in cultivation experiments are as described in this section unless otherwise stated. Appendix 1 contains a complete list of the suppliers of tissue culture materials.

2.7.1 Complete medium preparations: Complete medium preparations consisted of complex tissue culture medium, serum and supplements. The preparations tested are described in the materials and methods for each experiment and subsequently abbreviated as: the complex culture medium/concentration and type of serum, e.g. M199/40FBS.is Medium 199 with 40% foetal bovine serum.

Major supplements, other than 2mM l-glutamine and antibiotics, are included in the abbreviations.

All of the complete medium preparations for parasite cultures were routinely gassed with 5% CO_2 in air and stored at 4-6°C for a maximum period of ten days.

(a) Complex tissue culture media - All of the following complex tissue culture media were supplied by Gibco Europe in liquid form:

	Gibco Europe Catalogue No.	Abbreviation in thesis
Iscove's Modified Dulbecco's Medium	041-1980	IMD
L-15 (Leibovitz) Medium	041-1415	L-15
Medium 199 with Hanks' salts, 25mM HEPES buffer and l-glutamine	041-2350	M199
MEM-Minimum Essential Medium (Eagle) with Earle's salts and 25mM HEPES buffer, without l-glutamine	041-2360	MEM-E
MEM-Minimum Essential Medium (Eagle) with Hanks' salts and 25mM HEPES buffer, without l-glutamine	041-2370	MEM-H
MEM-Alpha Medium	041-2571	MEM-Alpha
NCTC 135 Medium	041-1350	NCTC 135
RPMI 1640 with 25mM HEPES buffer and l-glutamine	041-2400	RPMI 1640

(b) Sera - Foetal bovine serum (FBS) was obtained from Gibco Europe and unless otherwise indicated was heat inactivated by incubation at 56°C for 30 minutes. Normal bovine serum (NBS) and pre-infection serum (PIS) were removed from blood which was incubated at 37°C for one to two hours, placed at 4-6°C for four to eight hours

and then centrifuged at 2500-3000 x g for 25 minutes. Fresh autologous serum was removed from the defibrinated blood of T. annulata-infected calves after centrifugation at 1000 x g for ten minutes (5°C), and filtered through a 1.2 μ MF filter (Millipore).

(c) Medium supplements - L-glutamine (Gibco Europe) was routinely added to give a concentration of 2mM in all of the final complete media. Standard antibiotic concentrations per ml of the complete media for T. annulata-infected lymphoid cell cultures and B. bovis cultures were 100 iu sodium benzylpenicillin (Crystapen, Glaxo Lab.) and 100 μg streptomycin sulphate (Evans Medical). Theileria piroplasm culture media were supplemented with 50 μg/ml of gentamycin sulphate (Shering Corp., U.S.A.).

The hypoxanthine (G-hydroxypurine, Sigma Chemical), reduced glutathione (Sigma Chemical) and D-glucose (Analar grade - BDH Chemicals) were supplied as crystalline powders which were dissolved in medium and sterilized by filtration through a 0.22 μ MF filter (Millipore). The pH of the glutathione solution was titrated to 7.0 with 1N NaOH (BDH Chemicals) before filtration.

2.7.2 Culture vessels: The sterile, disposable plastic tissue culture vessels used are referred to as microtitre wells, 2 cm^2 wells and flasks. Microtitre wells were 6.4 mm diameter, flat-bottomed wells with a culture surface area of 0.3225 cm^2 (96-well Nunclon^R microtest plate with a lid, Nunc, Gibco Europe). The 2 cm^2 flat-bottomed wells had a 16 mm diameter and a 2 cm^2 surface area (24-well Tissue Culture Cluster^{24} plate with lid, Costar). The tissue culture flasks had screw-tight caps and a 25 cm^2 culture surface area when placed horizontal (Nunc, Gibco Europe).

2.8 Establishment and Maintenance of Piroplasm Cultures

2.8.1 Bovine aortic endothelial monolayers: The bovine aortic endothelial monolayers (BAE) were established at the CTVM in 1980 (C.G.D. Brown, personal communication). BAE cell lines, intended for use as feeder layers, were passaged one to three days prior to the establishment of piroplasm cultures. The overlying 5 ml of complete medium was removed from a culture with a confluent monolayer and the cells rinsed with phosphate buffered saline (PBS - Dulbecco A, Oxoid). Three ml of a 0.02% (w/v) solution of the disodium salt of ethylene diamine tetraacetic acid (BDH Chemicals) in PBS was added to the culture flask. After incubation at 37°C for two to seven minutes, 7 ml of complete medium was added, the detached cells resuspended and the suspension centrifuged at 500 x g. The pellet was resuspended in 5 ml complete medium and 0.5 ml aliquots of a 1:10 dilution of the cell suspension deposited into 2 cm^2 wells.

2.8.2 Preparation of erythrocyte suspensions: Cattle were bled by jugular puncture with a 14 gauge 1½ inch needle. Free flowing blood was collected aseptically either into 50 ml tubes with glass beads and defibrinated by shaking, or into 75-250 ml Ehrlenmeyer flasks in which defibrination was achieved by stirring with three wooden sticks.

Defibrinated blood from cattle infected with Theileria or Babesia was centrifuged for ten minutes at 1000 x g (15°C) and the serum and upper 20% of cells removed. The concentrated erythrocytes were aspirated from the bottom of the tube and used to prepare erythrocyte suspensions in complete medium for piroplasm cultures.

Defibrinated blood from normal, uninfected cattle was centrifuged at 2800 x g for 25 minutes (5°C) and the serum and upper 20% of cells discarded. Normal bovine erythrocyte suspensions for addition to Theileria cultures were prepared directly from the concentrated erythrocytes. Erythrocytes for B. bovis cultures were transferred to fresh medium and recentrifuged at 1000 x g for ten minutes (5°C). The concentrated erythrocytes from the second centrifugation were used to prepare suspensions in complete B. bovis culture medium.

The concentration of the erythrocyte suspensions and the volumes used either to establish or add to cultures are given in each experimental description.

2.8.3 Maintenance of piroplasm cultures: Tissue culture plates were gassed either in plastic self-sealing boxes (incubation boxes) or in modified Mackintosh Fildes jars. The Mackintosh Fildes jars (Rodwell Scientific Instruments) were mounted horizontally with aluminium wire and springs on metal stands. A metal shelf was fixed inside each jar and a 25 mm Swinnex filter (Millipore) packed with sterile, absorbent cotton wool attached by rubber tubing to the inflow valve. Plastic petri dishes contaning sterile distilled water were placed in incubation boxes and jars to maintain humidity.

Cultures in flasks or incubation boxes were routinely gassed with 5% CO_2 in air (British Oxygen). Reduced oxygen tensions were achieved by two methods using the modified Mackintosh Fildes jars:

(a) Atmospheres of 3% or 10% oxygen, with 10% CO_2 and the remainder nitrogen, were created by a gas evacuation/replacement technique. A vacuum pressure (Speedivac pressure pump-W. Edwards and Co., London) of either 380 or 650 mm of mercury was applied to

evacuate approximately 50% or 85.5% of the air from the sealed jars. The air was replaced with nitrogen from a rubber bladder reservoir. A water pressure vacuum system with a mercury pressure monitor was used to evacuate 10% of the equilibrated atmosphere which was replaced with CO_2.

(b) Airtight jars were flushed with a gas mixture of 12% oxygen, 5% CO_2 and 83% nitrogen (British Oxygen) at regulator setting 5 for three to five minutes before closing the outflow and inflow valves.

After gassing all cultures were incubated at 37°C in a water-jacketed incubator (Laboratory Thermal Equipment). The complete medium overlying the settled erythrocyte layer in piroplasm cultures was replaced daily with fresh complete medium.

2.9 Evaluation of Erythrocyte Invasion

A fluorescein stain solution was prepared by dissolving 40 mg of fluorescein isothiocyanate (Sigma Chemical) in 20 ml of PBS, and filter sterilized.

Uninfected erythrocytes were obtained from an adult bovid and concentrated as described in Section 2.8.2. A 0.5 ml aliquot of concentrated erythrocytes was added to 5 ml of fluorescein stain solution and incubated for 30 minutes at 4-6°C. The unreacted stain was removed by washing four times in PBS and once in M199.

Separate 10% (v/v) suspensions of concentrated fluorescein-stained and unstained erythrocytes were prepared in complete medium consisting of 60% M199 and 40% serum, either FBS or NBS. Suspensions of 10% (v/v) concentrated erythrocytes, obtained from the blood of T. annulata-infected cattle as described in Section 2.8.2, were

prepared in either M199/40 FBS or M199/40 NBS and mixed 1:2 with suspensions of unstained or fluorescein-stained erythrocytes in the corresponding complete media.

Cytocentrifuge smears were prepared from each of the suspensions before dispensing in 0.2 ml aliquots into replicate microtitre wells. Microtitre plates were placed in culture incubation boxes, gassed with 5% CO_2 in air, and maintained as in Section 2.8.3.

At 24 hour intervals duplicate cytocentrifuge smears were prepared (Section 2.12.1) from two separate wells for each suspension, air dried and fixed with methanol. One smear was stained for five minutes with dilute ethidium bromide (Boots Chemical) in PBS, rinsed with Giemsa buffer, air dried and examined under oil at x 54 magnification on a fluorescence microscope (Orthoplan, Ernst Leitz Wetzlar) with BG38 and GG475 primary, K530 suppression and KP500 interference filters. The second smear was stained with Giemsa as described in Section 2.12.1 and the percentage of infected erythrocytes determined. The number of parasites within 200 parasitized erythrocytes (PRBC) and the PRBC per 1000 erythrocytes were counted on smears from samples of each suspension on day 0 and duplicate cultures on day 6.

2.10 Establishment of Theileria Cultures from Tissue Biopsies

2.10.1 Biopsy techniques: Tissue biopsy samples were obtained by the following procedures and deposited directly into 10 ml of complete medium consisting of RPMI 1640 with 20% FBS (RPMI 1640/20 FBS), with 10 iu/ml of preservative free lithium heparin (Grade IV, Sigma Chemical):

(a) Lymph node biopsy samples were aspirated through a 14 gauge 1½ inch needle into a syringe containing 5 ml of medium with heparin.

(b) Splenic tissue samples, 1 cm^3, were obtained aseptically by incision after surgical splenectomy.

(c) The liver biopsies were taken with a Downer liver biopsy needle inserted through a stab puncture made at either the 11th or 12th intercostal space, 8 cm ventral to the transverse processes of the ribs on the right side.

2.10.2 Culture establishment: Each biopsy sample was manually disrupted and then centrifuged at 200 x g for five minutes (20°C). All but 2 ml of the supernate was removed, the cells resuspended in 6 ml of cold (5°C) 0.17M ammonium chloride in PBS and left at 5°C for ten minutes. The suspension was underlayered with 10 ml of complete medium and centrifuged at 200 x g for five minutes (20°C). The pellet was resuspended in 5 ml RPMI 1640/20 FBS, and the suspension deposited into a flask, gassed with 5% CO_2 in air and incubated at 37°C. The medium was changed as required, at least three times each week.

2.11 Establishment of Theileria Cultures from Peripheral Blood

The method described is a modification of a lymphocyte isolation technique (Shortman, Williams and Adams, 1972). Blood collected in 10 ml vacutainer tubes with 143 U.S.P. units of lithium heparin (Becton-Dickinson, U.K.) was centrifuged at 1800 x g for 20 minutes (5°C). The supernatant plasma was removed and retained. The buffy coat was removed in 1 ml, resuspended in 4 ml of cold (5°C)

PBS and mixed with 15 ml of a cold (5°C) solution of 0.17M ammonium chloride in PBS. The suspension was placed at 5°C for ten minutes, underlayered with 5-7 ml autologous plasma and centrifuged at 200 x g (20°C) for seven minutes. The pellet was resuspended in 6 ml of RPMI 1640/20 FBS and deposited in 1 ml aliquots into six 2 cm^2 wells. Cultures were placed in humidified incubation boxes and gassed with 5% CO_2 in air. Fresh medium was added as required, at least three times weekly.

2.12 Techniques for the Examination of Parasite Samples

2.12.1 Light microscopy: Smears of culture samples were prepared on glass microscope slides (Chance Propper) using a cyto-centrifuge (Cytospin, Shandon Southern Instruments). Slides were moistened with a five minute prespin (1000 rpm) of complete medium before 50-100 µl samples of Theileria-infected lymphoid cell cultures were centrifuged for ten minutes at 1000 rpm. Piroplasm culture samples, in 10-30 µl volumes, were centrifuged for five minutes at 700 rpm without a prespin.

Cytocentrifuge and routine blood smears from cattle were air dried, fixed with methanol and stained for 40 minutes in a 5% (v/v) dilution of Giemsa stock solution (Appendix 2) in aqueous buffer (pH 7.2). Smears were rinsed with buffer, air dried and examined under oil at x 500 or x 1000 magnification using compound light microscopes (Ortholux or Dialux 20, Ernst Leitz Wetzlar).

2.12.2 Transmission electron microscopy: The preparation of reagents in described in Appendix 2. The cacodylate buffer used was a standard 0.2M solution, pH 7.3. The glutaraldehyde fixative

was a 2.5% (v/v) solution in cacodylate buffer, supplemented with 2mM calcium chloride. The osmium tetroxide (OsO_4) fixative was a 1% (w/v) solution of OsO_4 in cacodylate buffer.

Lymph node biopsy samples were deposited directly into the glutaraldehyde fixative, placed at 4-6°C for two hours, washed four times with cacodylate buffer and post-fixed for 45 minutes in OsO_4 fixative. Aliquots of 2-5 ml defibrinated blood or piroplasm culture suspensions were centrifuged at 500 x g for ten minutes (15°C), the pellets fixed for one to two hours with glutaraldehyde fixative and then washed four times with cacodylate buffer. After post-fixing for one hour in the OsO_4 fixative the samples of parasitized erythrocytes were stained for one hour, in the dark, with a saturated solution of uranyl acetate (EM Scope Lab.) in 50% ethanol.

Two methods were employed for the subsequent dehydration, araldite impregnation and polymerization of samples:

(a) After the initial uranyl acetate stain, samples from T. annulata (Hissar) piroplasm cultures were rinsed with cacodylate buffer and dehydrated in a graded alcohol series of 10, 50, 70, 90 and 100% (v/v) ethanol. The pellets were immersed for 15 minutes in two changes of propylene oxide (Epoxypropane, BDH Chemicals) placed for one hour in a 1:1 mixture of araldite (Agar Aids) and propylene oxide, and left overnight in pure araldite. The next day the samples were embedded in fresh araldite and polymerized at 60°C for two to three days.

(b) All other samples were rinsed with cacodylate buffer and immersed for ten minute periods in dilutions of 50, 70 and 90% (v/v)

acetone, followed by four changes of 100% acetone (Analar grade, BDH Chemicals). An equal volume of araldite (Agar Aids) was added to the final acetone wash and the samples left overnight at 60°C in an open container. Samples were placed on a processing wheel (EM Scope Lab.) for the final embedding procedure which involved, at one hour intervals, two changes of araldite without accelerator and two changes of araldite with accelerator. Samples were embedded in fresh araldite (with accelerator) and polymerized at 60°C for a minimum of 48 hours.

Silver sections, of 60-90 nm, were cut from the blocks with an ultramicrotome (Omu, Reichert), placed on copper grids and stained with a saturated solution of uranyl acetate in 50% ethanol for 30 minutes. After several rinses in 50% ethanol, sections were stained for five minutes with Reynolds lead citrate (Reynolds, 1963) and rinsed with distilled, deionized water. Stained sections were viewed on a transmission electron microscope (Phillips 400, Pye Unicam) and photographed on electron image film (Kodak).

2.13 Techniques for Radioisotope Incorporation Studies

2.13.1 Isotope preparation and addition to cultures: The tritiated nucleic acid precursors employed were supplied by Amersham International and are listed in Table 2.2.

The hypoxanthine isotope was supplied as a lyophylate with an activity of 1.0 mCi per ampoule. The contents of a single ampoule were dissolved in 2.0 ml of 50% ethanol, dispensed in 0.2 ml aliquots (100 µCi) and stored at -20°C. All of the other isotopes, supplied as sterile aqueous solutions with activities of 1 mCi/ml, were dispensed in 0.1 ml (100 µCi) aliquots and stored at 4-6°C.

Table 2.2 Tritiated nucleic acid precursors supplied by Amersham International

	Code	Batch	Specific Activity Curies/mmol	mCi/mg
Purine Precursors				
(2-^{3}H) Adenine	TRK 311	45	23	170
(2-^{3}H) Adenosine	TRK 423	39	22	82
(8-^{3}H) Guanosine	TRK 222	45	5	17.7
(G-^{3}H) Hypoxanthine	TRA 74	27	5.7	42
Pyrimidine Precursors				
(5-^{3}H) Cytidine	TRK 198	73	30	122
(6-^{3}H) Thymidine	TRK 61	179	26	107
(6-^{3}H) Uridine	TRK 250	36	22.6	93

Before each experiment the ethanol in the hypoxanthine and adenine solutions was evaporated under a flow of hot air. The contents of a single vial of the required isotope were then added to 4.9 ml of MEM-H. Each of the 1:50 dilutions of isotope in medium, with an activity of 20 µCi/ml, was added in 25 µl volumes to the designated piroplasm cultures in microtitre wells to give a final radioactive concentration of 0.5 µCi per well.

2.13.2 Evaluation procedures: Cytocentrifuge smears were prepared from sample wells, fixed with methanol, stained with Giemsa and examined as for piroplasm cultures (Section 2.12.1). Counts were made of the number of parasitized erythrocytes per 1000 erythrocytes in samples from cultures with and without isotopes.

The incorporation of tritiated compounds was quantitatively assessed by liquid scintillation counting after a 24 hour incubation period. Cultures were harvested with a semi-automated cell harvester (Titertek Cell Harvester, Skatron) onto microfibre filters (Titertek, Skatron; Whatman). Filter paper discs were dried for 24 hours and deposited into glass counting vials with 10 ml PPO-POPOP based scintillation fluid (Appendix 2). Vials were counted for two minutes each in a scintillation counter (Packard Instrument).

2.14 Statistical Methods

The determination of mean values, Student's t-test, analysis of variance, Poisson distribution analysis and Chi-square test were conducted as described by Snedecor and Cochran (1980).

CHAPTER THREE

IN VITRO CULTIVATION OF THEILERIA ANNULATA

3.1 Introduction

Initial studies on the cultivation of theilerial piroplasms were conducted with Theileria annulata because, unlike T. parva, intraerythrocytic multiplication is an accepted component of the parasite's life cycle in cattle (Dschunkowsky, 1927; Sergent et al., 1945; Neitz, 1957; Barnett, 1968). Different culture environments were tested in a series of five experiments to determine if:

(a) T. annulata would multiply, and if so by what mode, within the erythrocytes in stationary cultures;

(b) parasites produced by intraerythrocytic multiplication would invade uninfected erythrocytes in vitro; and

(c) any of the environmental factors tested would favour parasite propagation.

The basic methodology employed was inspired by the cultivation systems developed for Plasmodium falciparum (Trager and Jensen, 1976) and Babesia bovis (Levy and Ristic, 1980). The rationale for testing various culture factors including reduced oxygen tensions, erythrocyte concentrations, sera, media, medium supplements and the presence of bovine aortic endothelial monolayers will be briefly discussed.

Reduced oxygen tensions, shown to be beneficial in the cultivation of intraerythrocytic protozoa, have been achieved by incubation in candle jars (Trager and Jensen, 1976; Jensen and Trager, 1977; Väyrynen and Tuomi, 1982) by administration of a continuous flow of gas with 1-10% oxygen (Trager and Jensen, 1976; Siddiqui, 1979; Butcher,

1979; Siddiqui and Palmer, 1981) or by flushing a closed vessel with gas mixtures of 5-10% oxygen (Chin, 1979; Butcher, 1981). Atmospheres with reduced oxygen tensions were created for T. annulata piroplasms in modified Mackintosh Fildes jars which held multiple tissue culture plates.

The depth of the settled erythrocyte layer and overlying medium in cultures affect gas diffusion (McLimans, 1972). Levy and Ristic (1980) proposed that metabolizing B. bovis, when present in sufficient numbers, could create a microaerophilic environment within the stationary erythrocyte layer in MASP cultures which were gassed with 5% CO_2 in air. Erythrocyte suspensions of 10%, dispensed into microtitre wells to give a culture depth of 0.62 cm,as recommended for MASP cultures, were initially tested in T. annulata cultures. Subsequently, lower concentrations of erythrocytes, similar to those used in Plasmodium cultures (Trager and Jensen, 1976; Trager, 1982) were used, with the intention of stimulating growth by reducing the parasite concentration (Trager and Jensen, 1978; Trager, 1979).

Growth of virtually all types of cells (Barnes and Sato, 1980) and many parasites (Ryley and Wilson, 1978) in vitro depends on the presence of serum in the culture medium. Medium with foetal bovine serum (FBS) supported the growth of P. falciparum (Haynes, Diggs, Hines and Desjardins, 1976; Jensen, 1979) and was comparable to medium supplemented with human serum after heat inactivation (Zhengren et al., 1980) or additional supplementation (Siddqui, 1979; Siddiqui and Palmer, 1981). In contrast, FBS was shown to be toxic to B. bovis in vitro (Levy, Clabaugh and Ristic, 1982) while normal adult bovine sera (NBS), at a concentration of 40% of the complete medium, was

propitious (Erp et al., 1980a; Levy and Ristic, 1980). Several sera including FBS with and without heat inactivation, NBS, autologous preinfection sera and homologous preinfection sera were evaluated in T. annulata cultures. In addition, autologous sera were collected from infected cattle on the same day as the parasitized erythrocytes were taken for culture establishment and added to the complete medium to see if these sera had a stimulatory effect on parasite multiplication.

Complex tissue culture media were compared in an attempt to find a formulation with the optimal nutrient composition. The media, MEM, L-15 and RPMI 1640, used routinely in Theileria-infected lymphoid cell cultures (Brown, 1979a; Munderloh and Kurtti, 1982), were tested in the piroplasm cultures. RPMI 1640, designed as a human leucocyte culture medium (Moore, Gerner and Franklin, 1967) has been used to grow P. coatneyi and P. falciparum (Trager, 1971; Trager and Jensen, 1976) and remains the preferred medium for the cultivation of the intraerythrocytic stages of P. falciparum (Trager and Jensen, 1980; Siddiqui and Palmer, 1981). Medium 199 has been utilized successfully for the cultivation of P. falciparum (Haynes et al., 1976; Zhengren et al., 1980), B. bovis (Erp et al., 1980a; Levy and Ristic, 1980) and B. divergens (Väyrynen and Tuomi, 1982; M. Pudney, personal communication; G. Konrad, personal communication).

The inclusion of purine bases and nucleosides is a distinctive feature of M199, MEM-Alpha and NCTC 135 media. Numerous studies have demonstrated that malarial parasites synthesize pyrimidines de novo and purines via salvage pathways with hypoxanthine as the major metabolite (Büngener and Nielson, 1967, 1969; Walsh and Sherman, 1968;

Sherman, 1977; Van Dyke, Trush, Wilson and Stealey, 1977; Webster and Whaun, 1981; Ting and Sherman, 1981). Radioisotope incorporation studies suggest that T. parva (Irvin, Boarer, Kurtti and Ocama, 1981) and several species of Babesia (Chiodini, 1973b; Irvin, Young and Purnell, 1978; Irvin and Young, 1979) utilize purine nucleotide precursors via similar pathways.

Supplementation with purine nucleic acid precursors, glucose and reduced glutathione has been shown to augment the growth of malarial parasites (Trager, 1943, 1950; Zolg et al., 1982) and prolong the viability of erythrocytes in vitro (Hogman, Hedlund and Zetterström, 1978; Strauss and de Verdier, 1980; Grimes, 1980). The potentially beneficial effects of these supplements justified testing them in T. annulata cultures. In addition, l-glutamine which is an essential metabolite in de novo pyrimidine synthesis (Lehninger, 1975) was supplemented because this compound is characteristically unstable in tissue culture media (Patterson, 1972).

Monolayers of slow growing adherent cells have been used as feeder layers that provide unidentified nutrient factors, beneficial to other cells and parasites cocultivated with the monolayers (Puck and Marcus, 1955; Brown, 1979a; Harel, 1981). A bovine aortic endothelial monolayer was tested as a feeder layer for T. annulata piroplasms, thus avoiding the introduction of cells from a different host species.

Differences exist between species, strains and even individual isolates of malarial parasites in the ease of culture establishment (Trigg, 1978; Butcher, 1981; D. Walliker, personal communication). To increase the chance of making a successful in vitro isolation of T. annulata, two parasite strains were tested and isolates made from two calves in each experiment.

3.2 Reduced Oxygen Tensions and Sera Tested in Ankara Strain Cultures

3.2.1 Experimental design: Blood was collected from calves 117 and 118 on days 10 and 11 post-infection with T. annulata (Ankara). Concentrated erythrocytes obtained as described in Section 2.8.2 were used to prepare 10% (v/v) erythrocyte suspensions in complete medium consisting of 60% M199 and 40% of the test serum.

Factors tested in this experiment were:

(a) Oxygen tensions of
  (i) 3% O_2
  (ii) 10% O_2

(b) Sera
  (i) Foetal bovine serum
  (ii) Homologous preinfection sera collected from calves 117 and 118, and pooled.

Erythrocyte suspensions were dispensed in 0.2 ml aliquots into microtitre wells and the microtest plates placed in modified Mackintosh Fildes jars. Atmospheres were created by a gas displacement system and the cultures maintained as described in Section 2.8.3.

Cultures were evaluated by counting the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes on Giemsa stained cytocentrifuge smears prepared at 48 hour intervals from two wells for each test factor.

3.2.2 Results: The means and standard deviations of the parasitized erythrocyte counts are summarised in Table 3.1. There was no significant change in the number of parasitized erythrocytes throughout the 8-day period in any of the cultures. The counts from each day were therefore pooled for display in Table 3.1.

Table 3.1 Number of parasitized erythrocytes per 1000 erythrocytes in T. annulata (Ankara) cultures in Experiment 3.2

Day in vitro	Culture Samples			
	Blood taken day 10 post infection		Blood taken day 11 post infection	
	Calf 117	Calf 118	Calf 117	Calf 118
0	40 ± 7	38 ± 4	142 ± 2	196 ± 6
2	41 ± 7	33 ± 4	140 ± 8	195 ± 20
4	34 ± 7	31 ± 4	147 ± 8	197 ± 20
6	38 ± 5	33 ± 7	138 ± 9	192 ± 23
8	39 ± 8	36 ± 4	143 ± 26	188 ± 13

Figures are the mean ± standard deviation of the pooled counts from 8 samples.
(i.e. 2 wells sampled for each factor x 4 test factors)

The morphology of the parasites did not change appreciable during the culture period.

3.3 Methods for Erythrocyte Suspension Preparation, Complex Media and Sera Tested in Hissar Strain Cultures

3.3.1 Experimental design: Blood was collected from calves 121 and 122 on days 11 and 13 post-infection with T. annulata (Hissar).

Factors tested in this experiment were:

(a) Methods for the preparation of erythrocyte suspensions

(i) Concentrated erythrocytes were prepared as described in Section 2.8.2.

(ii) Concentrated erythrocytes prepared as in Section 2.8.2 were washed two times in medium with a final centrifugation of ten minutes at 1000 x g.

The concentrated erythrocytes in (i) and (ii) were used to prepare 10% (v/v) suspensions in complete medium.

(iii) Defibrinated blood was mixed directly with an equal volume of complete medium.

(b) Complex tissue culture media - compared as 60% of the complete medium

(i) M199

(ii) MEM

(iii) RPMI 1640

(iv) L-15

(c) Sera - compared as 40% of the complete medium

(i) Foetal bovine serum

(ii) Autologous preinfection serum

(iii) Autologous serum collected on days 11 and 13 post-infection, at the same time as the blood was taken for culture establishment.

Parasitized erythrocyte suspensions in complete medium were dispensed in 0.2 ml aliquots into replicate microtitre wells, gassed with 5% CO_2 in air, and maintained as described in Section 2.8.3.

Cultures were evaluated by counting the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites in 100 PRBC in Giemsa stained cytocentrifuge smears from two cultures for each combination of test factors.

3.3.2 Results: Parasite counts made from the cultures established with blood taken on day 11 post infection, with parasitaemias between 1 and 4%, are displayed in Table 3.2. Counts of samples from cultures containing blood taken on day 13, with parasitaemias between 7 and 16%, are shown in Table 3.3.

At the time of culture establishment (day 0) the majority of the parasitized erythrocytes contained single piroplasms, with two or three piroplasms seen in a small proportion, less than 10%, of the cells. By day 3-6 *in vitro* clusters of four small parasites with centrally oriented basophilic nuclei and distinct cytoplasmic tails, appeared within the erythrocytes (Figures 3.1 and 3.2). These quadruplet forms were not seen in the fresh blood smears or erythrocyte suspensions on the day of culture establishment.

The increased incidence of erythrocytes containing four parasites in cultures with FBS or autologous sera (AS) is shown in Tables 3.2 and 3.3. The parasite counts were pooled for display in the tables according to the medium and serum (either FBS or AS) because there was no apparent difference in the parasite counts between cultures prepared by the different methods described in (a) or between cultures receiving complete media with the two types of autologous sera tested.

There was no meaningful change in the number of parasitized erythrocytes within the cultures after six days *in vitro* (Tables 3.2

Figure 3.1

Theileria annulata (Hissar) after 4 days in stationary erythrocyte cultures established with blood from calf 121 collected on day 11 post infection in experiment 3.3 are shown in Figures 3.1 and 3.2. (x 1600)

Figure 3.2

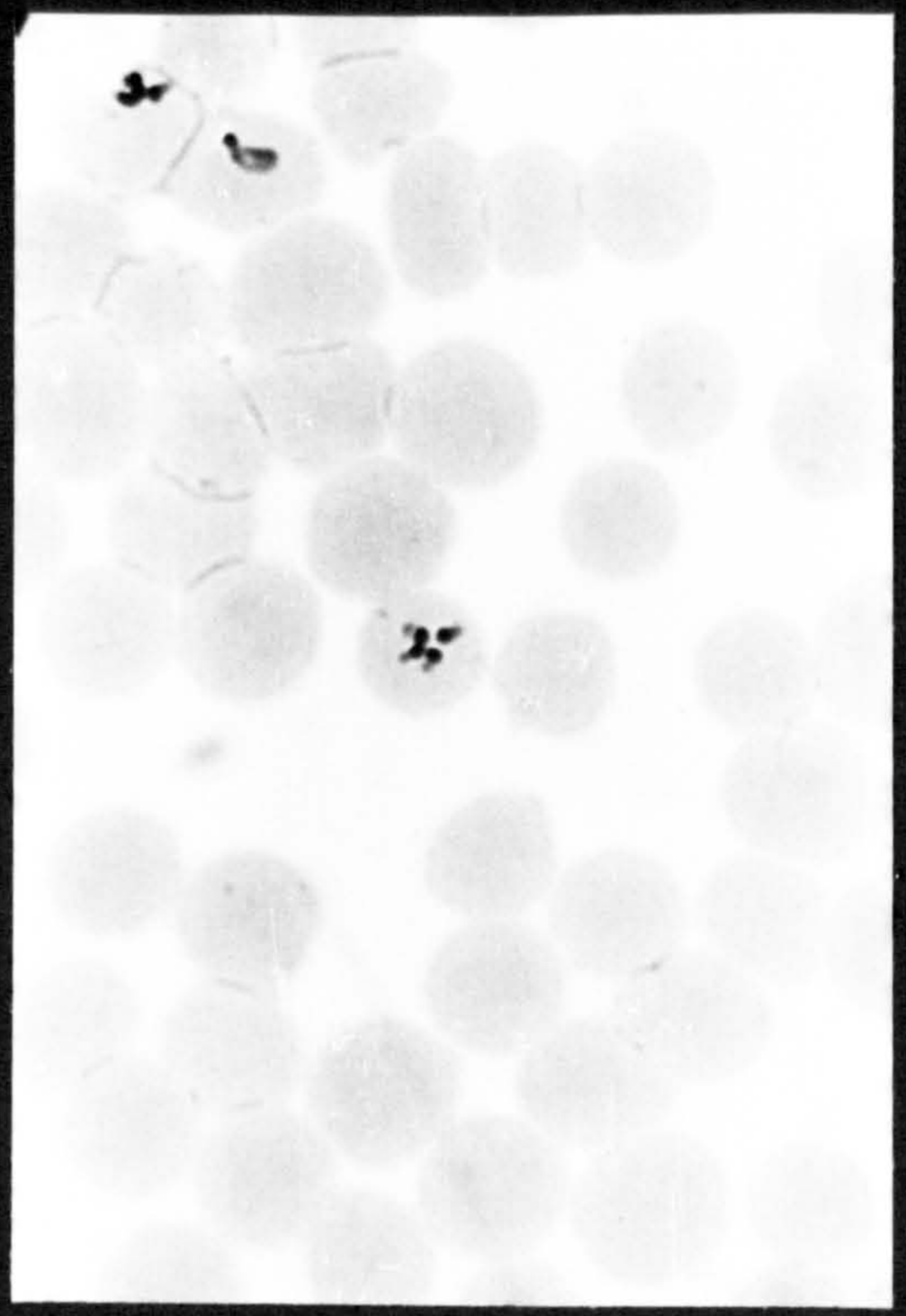


Table 3.2 Cultures established with blood taken day 11 post infection with Theileria annulata (Hissar) in Experiment 3.3

Number of parasitized erythrocytes (PRBC) per 1000 erythrocytes arranged according to the number of parasites in each PRBC

Sample			Calf 121							Calf 122						
Sera in complete media	Day in vitro	Medium	Number of parasites in erythrocyte 1	2	3	4	5	6	PRBC per 1000 erythrocytes	Number of parasites in erythrocyte 1	2	3	4	5	6	PRBC per 1000 erythrocytes
	0	Inocula pooled	20	1	0	0	0	0	21	33	1	0	0	0	0	34
Foetal bovine sera	6	M199	9	1	0	11	0	0	21	24	1	1	5	0	0	31
		MEM-H	14	0	0	5	0	0	19	21	1	0	8	0	0	32
		RPMI 1640	14	0	0	4	0	0	18	24	0	0	8	0	0	32
		L-15	13	1	0	2	0	0	16	23	1	0	2	0	1	27
	0	Inocula pooled	21	1	0	0	0	0	22	33	1	0	0	0	0	34
Autologous sera	6	M199	24	0	0	2	0	0	26	30	1	0	0	0	0	31
		MEM-H	22	0	0	0	0	0	22	26	2	0	2	0	0	30
		RPMI 1640	17	1	0	0	0	0	18	26	2	0	0	0	0	28
		L-15	15	1	0	0	0	0	16	30	0	0	2	0	0	32

Figures are the mean counts of 8 samples on day 0 and samples from 2 cultures for each medium/serum combination after 6 days in vitro.

Table 3.3 Cultures established with blood taken day 13 post infection with Theileria annulata (Hissar) Number of parasitized erythrocytes (PRBC) per 1000 erythrocytes arranged according to the number of parasites in each PRBC

Sample			Calf 121							Calf 122						
Sera in complete media	Day in vitro	Medium	Number of parasites in erythrocyte 1	2	3	4	5	6	PRBC per 1000 erythrocytes	Number of parasites in erythrocyte 1	2	3	4	5	6	PRBC per 1000 erythrocytes
	0	Inocula pooled	82	3	0	0	0	0	85	144	7	4	0	0	0	155
Foetal bovine sera	6	M199	48	19	2	16	1	0	86	115	4	1	7	1	0	128
		MEM-H	64	6	1	19	0	0	90	108	6	2	24	0	0	140
		RPMI 1640	70	7	10	7	0	0	94	117	6	0	21	0	0	144
		L-15	68	2	1	7	0	0	78	118	8	0	14	0	0	140
Autologous sera	0	Inocula pooled	79	3	0	0	0	0	82	137	10	0	0	0	0	147
	6	M199	72	8	2	1	0	0	83	141	8	1	1	0	1	152
		MEM-H	79	8	2	1	0	0	90	121	11	2	2	0	0	136
		RPMI 1640	91	3	0	4	0	0	98	128	10	0	3	0	0	141
		L-15	69	2	0	1	0	0	72	136	6	0	1	0	0	143

Figures are the mean counts of 8 samples on day 0 and samples from 2 cultures for each medium/serum combination after 6 days in vitro.

Table 3.4 Comparison between counts of Theileria annulata (Hissar) in cultures with foetal bovine sera and autologous sera in the complete medium after 6 days in vitro.

Calf	Cultures established with blood taken day 11 post infection		Cultures established with blood taken day 13 post infection	
	F-value	Probability	F-value	Probability
121	11.60	$P < 0.01$	37.97	$P < 0.01$
122	6.35	$P < 0.05$	6.35	$P < 0.05$

Based on 2-way ANOVA of the total number of parasites per 1000 erythrocytes in 8 culture samples for each serum (df = 1,8).

and 3.3). Therefore, the total number of parasites counted per 1000 erythrocytes on day 6 was used to compare the media with either FBS or AS in a 2-way analysis of variance (ANOVA).

The following conclusions were based on this ANOVA:

(a) A comparison between the media tested showed that there was a significant difference in the number of parasites after six days in vitro in the cultures with different media which were established with blood taken from calf 121 either on day 11 ($F^3_8 = 8.24$, $P < 0.01$) or day 13 ($F^3_8 = 9.80$, $P < 0.01$) post-infection. The parasite counts were not, however, significantly different in the cultures established with blood from calf 122. In short, none of the media were consistently better than the other media tested.

(b) The number of parasites in cultures with FBS in the complete medium was consistently greater than in the corresponding cultures with autologous sera in the medium. The difference between the parasite counts in cultures with the two types of sera was significant as shown in Table 3.4.

The interaction between factors was not significant in this analysis of variance.

3.4 Oxygen Tensions, Complex Media and Erythrocyte Concentrations Tested in Hissar Strain Cultures

3.4.1 Experimental design: Blood was collected from calves 127 and 128 on day 10 post-infection with T. annulata (Hissar).

Factors tested in this experiment were:

(a) Oxygen tensions

(i) 12% O_2

(ii) air - approximately 19-20% O_2

(b) Complex tissue culture media - compared as 60% of the complete medium with 40% FBS

(i) M199

(ii) MEM-Alpha with 25 mm/ml HEPES buffer

(iii) RPMI 1640

(c) Erythrocyte concentrations

(i) 0.3%

(ii) 3.0%

prepared (v/v) with concentrated erythrocytes obtained as described in Section 2.8.2.

The erythrocyte suspensions in complete medium were dispensed in 1 ml aliquots into replicate 2 cm^2 wells for each test factor on duplicate plates. The tissue culture plates were placed in separate modified Mackintosh Fildes jars and gassed with mixtures of either 12% O_2, 5% CO_2 and 83% nitrogen, or 5% CO_2 in air. Cultures were maintained as described in Section 2,8.3.

Cultures were evaluated by counting the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites within 100 PRBC in Giemsa stained cytocentrifuge smears from two cultures for each combination of test factors every 48 hours. Day 0 counts were made from fresh blood smears.

3.4.2 Results: The means and standard deviations of the pooled counts of parasites in 100 PRBC made at 48 hour intervals are graphically illustrated in Figures 3.3 and 3.4. The same mean counts, plus parasitaemias, are tabulated in Appendices 3 and 4.

At the time of culture establishment the majority of the parasitized erythrocytes (PRBC) in the blood of calves 127 and 128, with parasitaemias of 1-2%, contained single piroplasms. The remaining PRBC had two or three parasites. By day 4-6 *in vitro* the incidence

of multi-parasitized erythrocytes increased dramatically (Figures 3.3 and 3.4). The greatest increase was in the incidence of erythrocytes with quadruplet forms, identical to those seen in Experiment 3.3, which appeared in all of the cultures. The number of erythrocytes with three parasites, morphologically similar to those in quadruplet forms, increased by day 6.

Five to 12 parasites were seen inside a small proportion of erythrocytes after day 4. The incidence of the latter multi-parasitized erythrocytes was proportional to the number of erythrocytes in the culture inocula which contained two or three piroplasms. At least one quadruplet form was always seen within erythrocytes containing a total of 5-12 parasites as seen in Figure 3.5.

The difference in the number of parasitized erythrocytes in the cultures after six days *in vitro*, compared to fresh blood smears on day 0, was not significant.

The results have been pooled for display because there were no apparently meaningful differences in the parasite counts from cultures with different test factors.

Based on this experiment, day 6 was selected as the standard day to sample replicate wells for comparisons between the test factors because the parasitaemias and incidence of multi-parasitized erythrocytes did not increase in subsequent evaluations (Figures 3.3 and 3.4, Appendices 3 and 4). Counts made from samples of two wells for each combination of test factors showed that there were no meaningful differences in the number of parasites between the cultures after six days *in vitro* (Appendices 5 and 6).

Parasites became progressively more pycnotic after day 10 *in vitro* and the cultures were discarded on day 14.

Figure 3.3 Theileria annulata (Hissar) cultures established with blood from calf 127 in experiment 3.4. Figures represent mean number of parasitized erythrocytes (PRBC) per 100 counted in 24 samples with specified number of parasities inside.

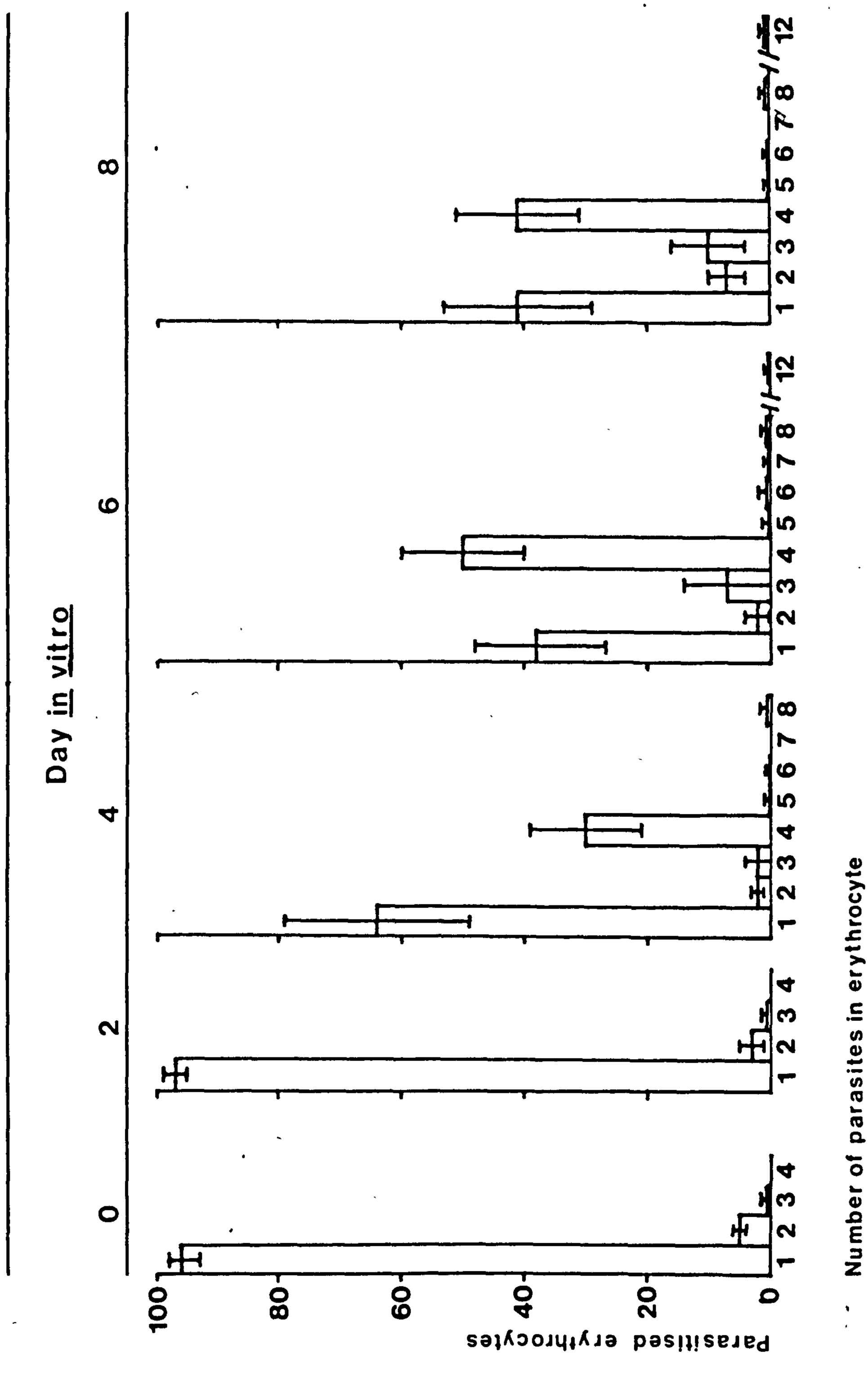

Number of parasites in erythrocyte
Parasitised erythrocytes
100
80
60
40
20
0
1 2 3 4
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8 9/12
1 2 3 4 5 6 7 8 9/12
Day in vitro
0
2
4
6
8

Figure 3.4 Theileria annulata (Hissar) cultures established with blood from calf 128 in experiment 3.4 Figures represent mean number of parasitized erythrocytes (PRBC) per 100 counted in 24 samples with specified number of parasites inside.

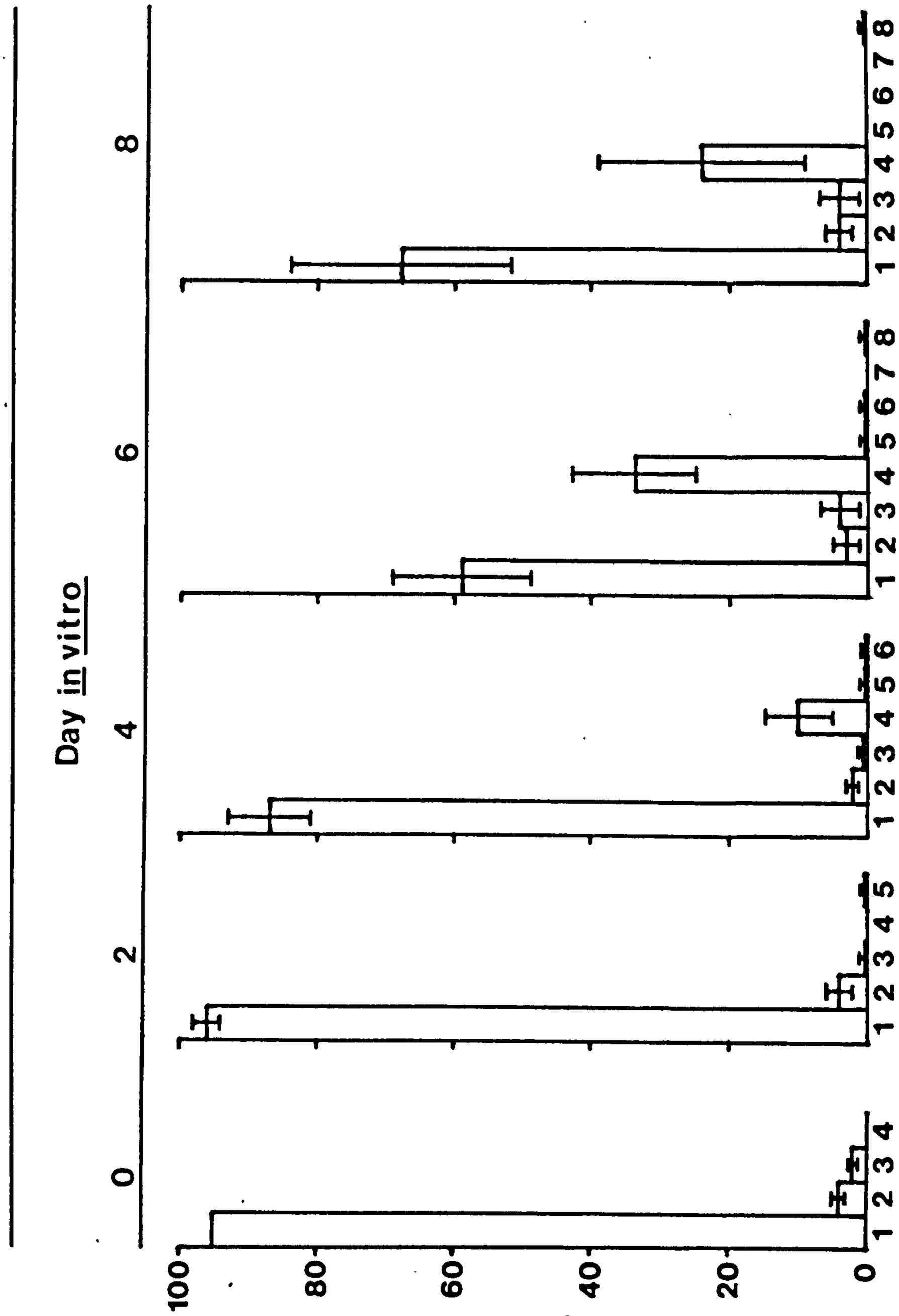
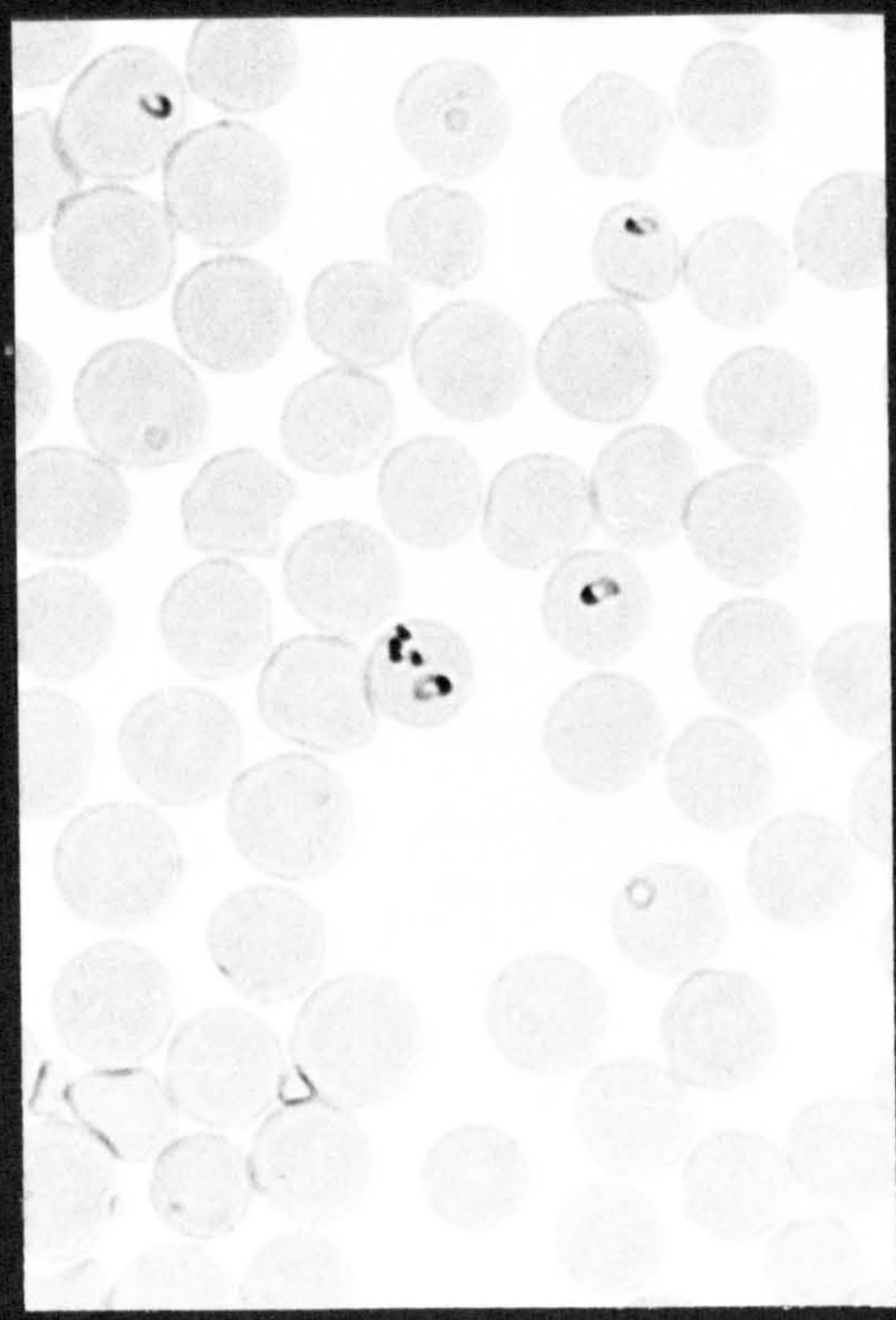

Parasitised erythrocytes
100
80
60
40
20
0
Number of parasites in erythrocyte
Day in vitro
0
2
4
6
8
1 2 3 4
1 2 3 4 5
1 2 3 4 5 6
1 2 3 4 5 6 7 8

Figure 3.5 Theileria annulata (Hissar) after 6 days in stationary erythrocyte cultures established with blood from calf 127.

(x 1600)

Erythrocytes with 5 parasites contained a single piroplasm and a quadruplet form.



3.5 Sera Concentrations, Medium Supplements and BAE Monolayers Tested in Hissar Strain Cultures

3.5.1 Experimental design: Blood was collected from calves 145 and 146 on day 10 post-infection with T. annulata (Hissar).

Factors tested in this experiment were:

(a) Foetal bovine serum concentrations of
   (i) 20%
   (ii) 40%
   with the remaining portion of the complete medium consisting of M199.

(b) Medium supplements - added per ml of M199/20 FBS
   (i) 20 mg glucose
   (ii) 50 µg hypoxanthine
   (iii) 50 µg hypoxanthine and 50 µg reduced glutathione (GSH)

(c) With and without bovine aortic endothelial monolayers (BAE) established as described in Section 2.8.1.

Suspensions of 3% (v/v) concentrated erythrocytes, obtained as described in Section 2.8.2,were prepared in the complete test media and dispensed in 1 ml aliquots into replicate 2 cm^2 wells for each combination of test factors. Cultures were gassed with 5% CO_2 in air and maintained as described in Section 2.8.3.

Cultures were examined at 48 hour intervals by inspection of Giemsa stained cytocentrifuge smears of the sampled wells. Counts were made from fresh blood smears on day 0 and from cytocentrifuge smears of two cultures for each combination of test factors, after six days in vitro. The number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites in 100 PRBC were counted in each smear.

3.5.2 Results: Day 6 counts were pooled for display in Figures 3.6 and 3.7 when the parasitaemia and total number of parasites per 100 PRBC were not significantly different. Parasite counts from fresh blood smears on day 10 post infection and cytocentrifuge smears of samples from two cultures for each combination of test factors after six days *in vitro* are given in Appendices 7 and 8.

The incidence of multi-parasitized erythrocytes, most notably with four parasites, increased in all of the cultures from day 0 to day 6 *in vitro*. The total number of parasitized erythrocytes did not, however, change significantly from the initial 2-4% parasitaemia during the cultivation period (Appendices 7 and 8).

The difference in the total number of parasites per 100 parasitized erythrocytes between day 0 and 6 *in vitro* was used as the basis for comparing the test factors in a 2-way ANOVA. There was no significant difference in the number of parasites between cultures with the various test factors which were established with erythrocytes from calf 145 (Figure 3.6, Appendix 7).

The difference in the parasite counts from samples of calf 146 cultures grown in media with different sera and supplements, with and without BAE monolayers was highly significant ($F^{4}_{10} = 28.7$, $P < 0.01$). The parasite increase was greatest in the 146 cultures with 40% FBS in the complete medium. Cultures grown on BAE monolayers in complete medium with 20% FBS (M199/20 FBS) supplemented with hypoxanthine and glutathione had a higher incidence of quadruplet forms, hence total number of parasites per 100 PRBC, than any of the other cultures with M199/20 FBS (Figure 3.7, Appendix 8).

The interaction between factors was not significant in this analysis of variance.

Figure 3.6 Theileria annulata (Hissar) cultures established with blood from calf 145 in experiment 3.5. Figures represent mean number of parasitized erythrocytes per 100 counted with specified number of parasites inside.

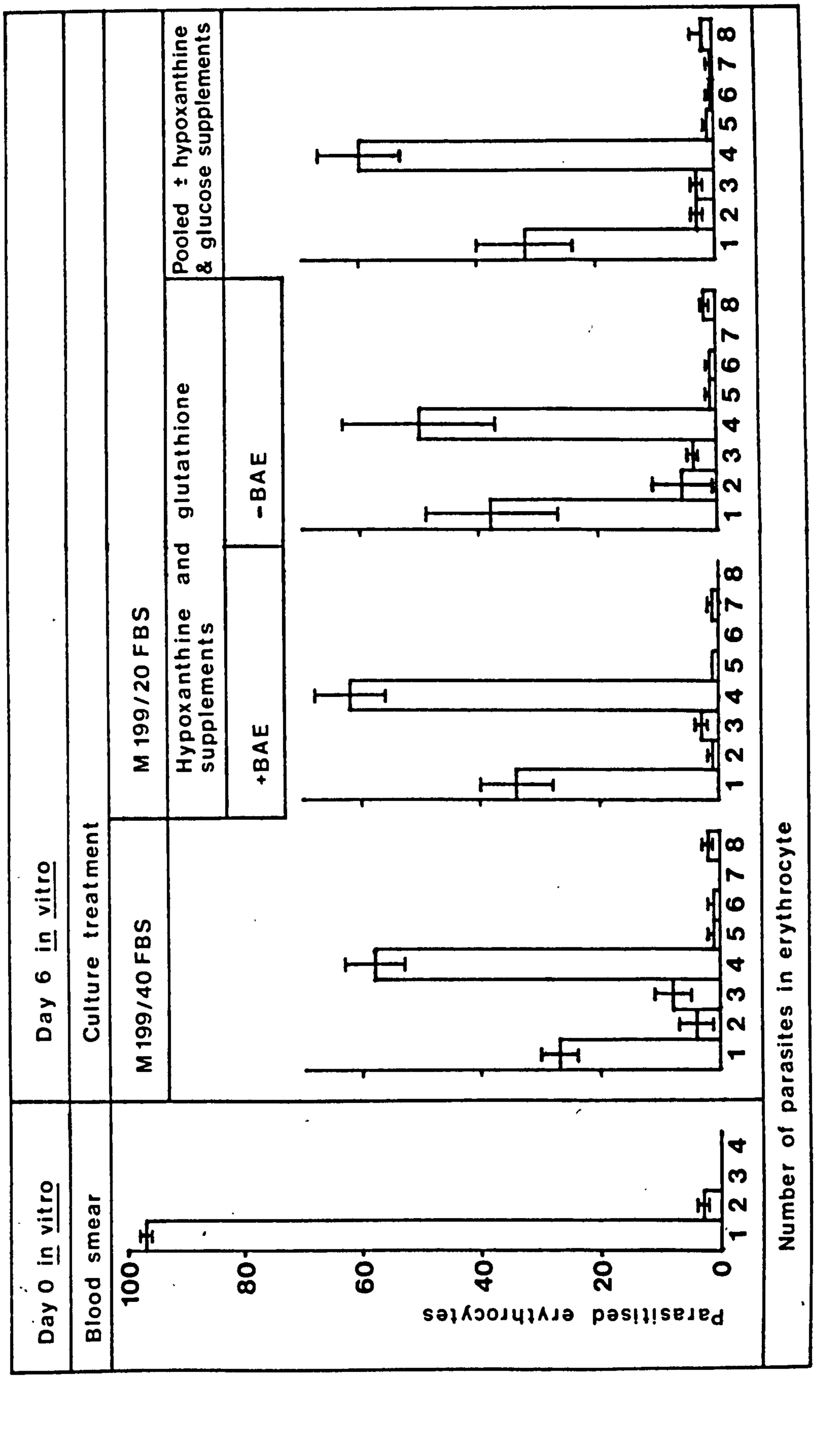
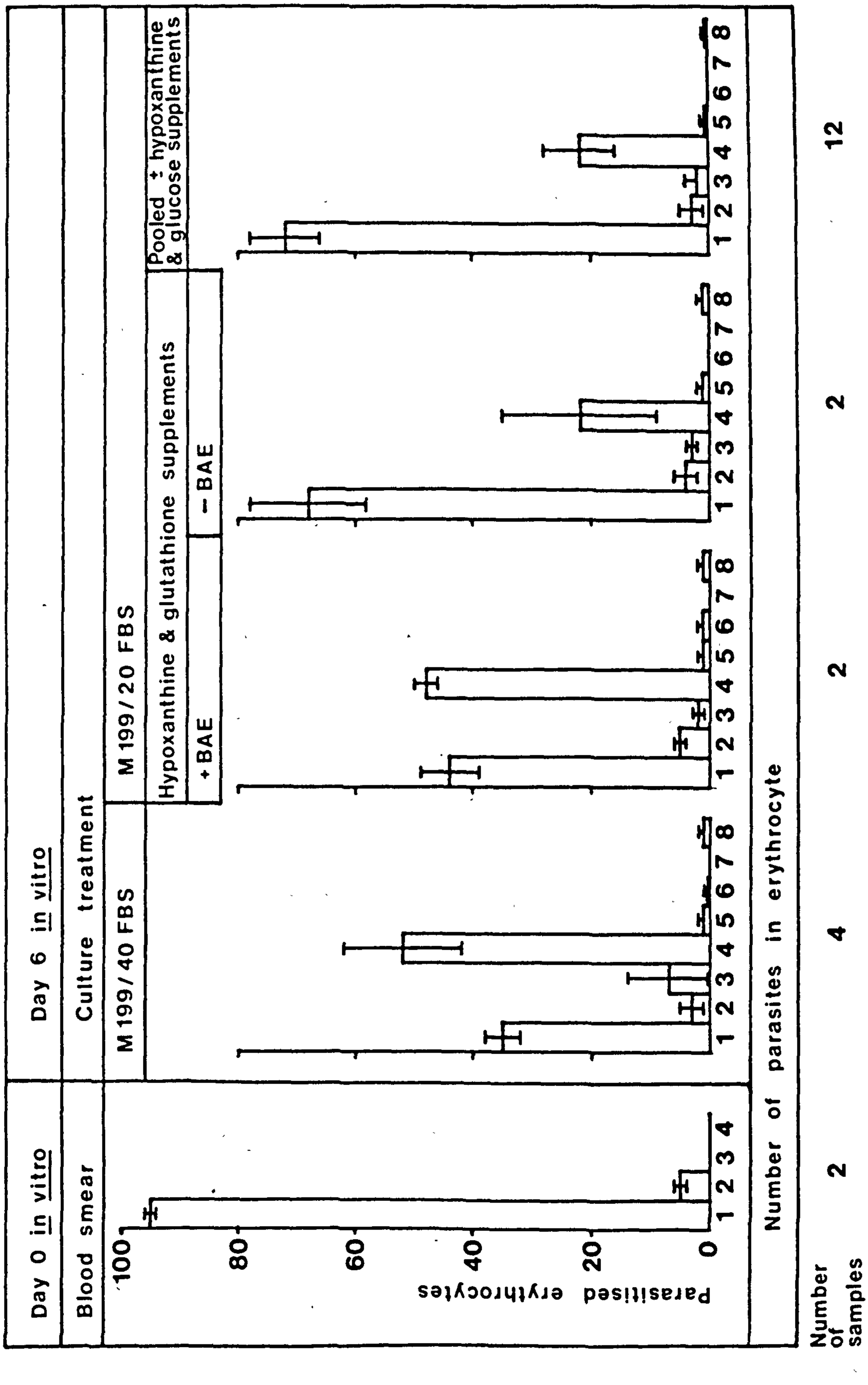

Day 6 in vitro
Culture treatment
M199/40 FBS
M199/20 FBS
Hypoxanthine and glutathione supplements
+BAE
-BAE
Pooled ± hypoxanthine & glucose supplements
Parasitised erythrocytes
100
80
60
40
20
0
1 2 3 4 5 6 7 8
Number of parasites in erythrocyte
Number of samples
12
2
2
4
Day 0 in vitro
Blood smear

Figure 3.7 Theileria annulata (Hissar) cultures established with blood from calf 146 in experiment 3.5. Figures represent mean number of parasitized erythrocytes per 100 counted with specified number of parasites inside.


Day 0 in vitro
Blood smear
Parasitised erythrocytes
100
80
60
40
20
0
1 2 3 4
Number of samples
2
Day 6 in vitro
Culture treatment
M 199 / 40 FBS
M 199 / 20 FBS
Hypoxanthine & glutathione supplements
+ BAE
- BAE
Pooled ± hypoxanthine & glucose supplements
1 2 3 4 5 6 7 8
12
2
2
4
Number of parasites in erythrocyte

The cultures were maintained for 14-21 days until the majority of the parasites became pycnotic.

3.6 Complex Media, Sera and Medium Supplements Tested in Ankara Strain Cultures

3.6.1 Experimental design: Blood was collected from calves 153 and 156 on day 10 post-infection with T. annulata (Ankara).

Factors tested in this experiment were:

(a) Complex media - compared as 80% of the complete medium with 20% FBS

(i) M199

(ii) MEM-Alpha with 25 mm/ml HEPES buffer added

(iii) NCTC 135 with 25 mm/ml HEPES buffer added

(b) Sera - compared as 40% of the complete medium with 60% M199

(i) Foetal bovine serum - routinely heat inactivated (FBS)

(ii) Foetal bovine serum - not heat inactivated (FBS^-)

(iii) Fresh adult normal bovine serum (NBS)

(iv) Autologous serum collected day 10 post infection (AS)

(v) Autologous serum collected day 14 post infection (AS)

(c) Medium supplements - added per ml of M199/20 FBS

(i) 50 µg reduced glutathione

(ii) 500 µg reduced glutathione

(iii) 10mM l-glutamine

Suspensions of 3% (v/v) concentrated erythrocytes, obtained as described in Section 2.8.2 were prepared in the complete test media and dispensed in 1 ml aliquots into replicate 2 cm^2 wells for each factor. Cultures were gassed with 5% CO_2 in air, and maintained as described in Section 2.8.3.

Cultures were examined at 48 hour intervals by inspection of Giemsa stained cytocentrifuge smears of the sampled wells. Counts

were made from fresh blood smears on day 0 and the cytocentrifuge smears from two cultures for each combination of test factors after six days *in vitro*. The number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites within 100 PRBC were counted in each smear.

3.6.2 *Results*: Figures 3.8 and 3.9 illustrate graphically the increase in the number of multi-parasitized erythrocytes in the cultures from calves 153 and 156 respectively, between day 0 and 6 *in vitro*. The mean values of parasite counts made from samples of cultures with 40% FBS, 20% FBS or 40% NBS or AS are summarised in Appendix 9.

Quadruplet forms, identical to those observed in experiments 3.3-3.5 appeared in all of the cultures. The greatest relative increase by day 6 *in vitro* was in the number of parasitized erythrocytes with four parasites (Figures 3.8 and 3.9). The incidence of erythrocytes with 5-8 parasites increased and quadruplet forms were always observed within these multi-parasitized erythrocytes.

There was no meaningful change in the number of parasitized erythrocytes during the culture period from the initial parasitaemias of 2-3% for calf 153 and 8-10% for calf 156.

Analysis of variance based on the difference in the number of parasites per 100 PRBC on day 0 and 6 *in vitro* showed that there was no significant difference between the complex media and various medium supplements tested. The only significant differences in the parasite counts in samples from calf 153 ($F^{4}_{9} = 30.16$, $P < 0.01$) and calf 156 ($F^{4}_{9} = 22.30$, $P < 0.01$) cultures were between the sera tested. The

Figure 3.8 Theileria annulata (Ankara) cultures established with blood from calf 153 in experiment 3.6. Figures represent mean number of parasitized erythrocytes per 100 counted with specified number of parasites inside.

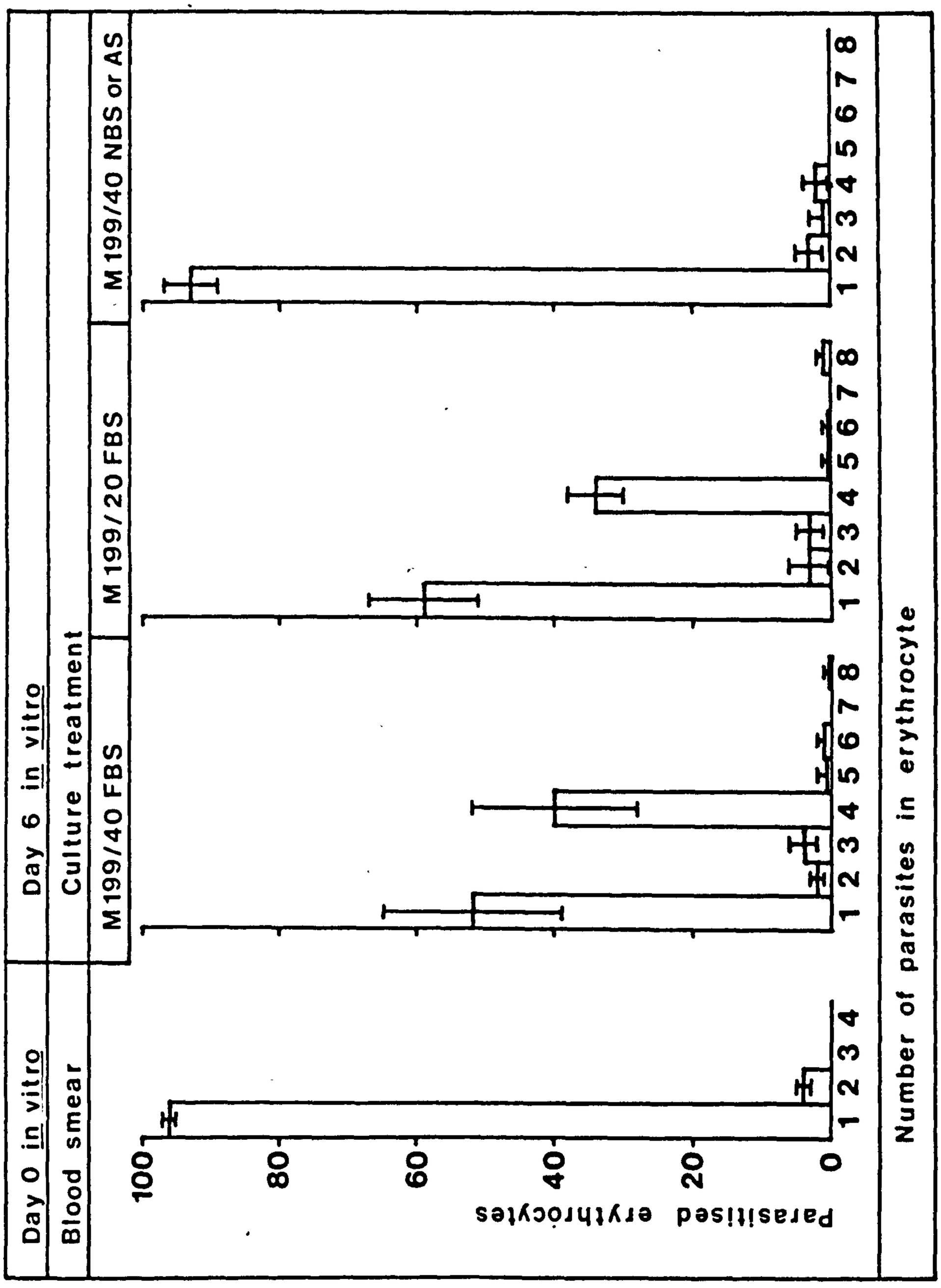
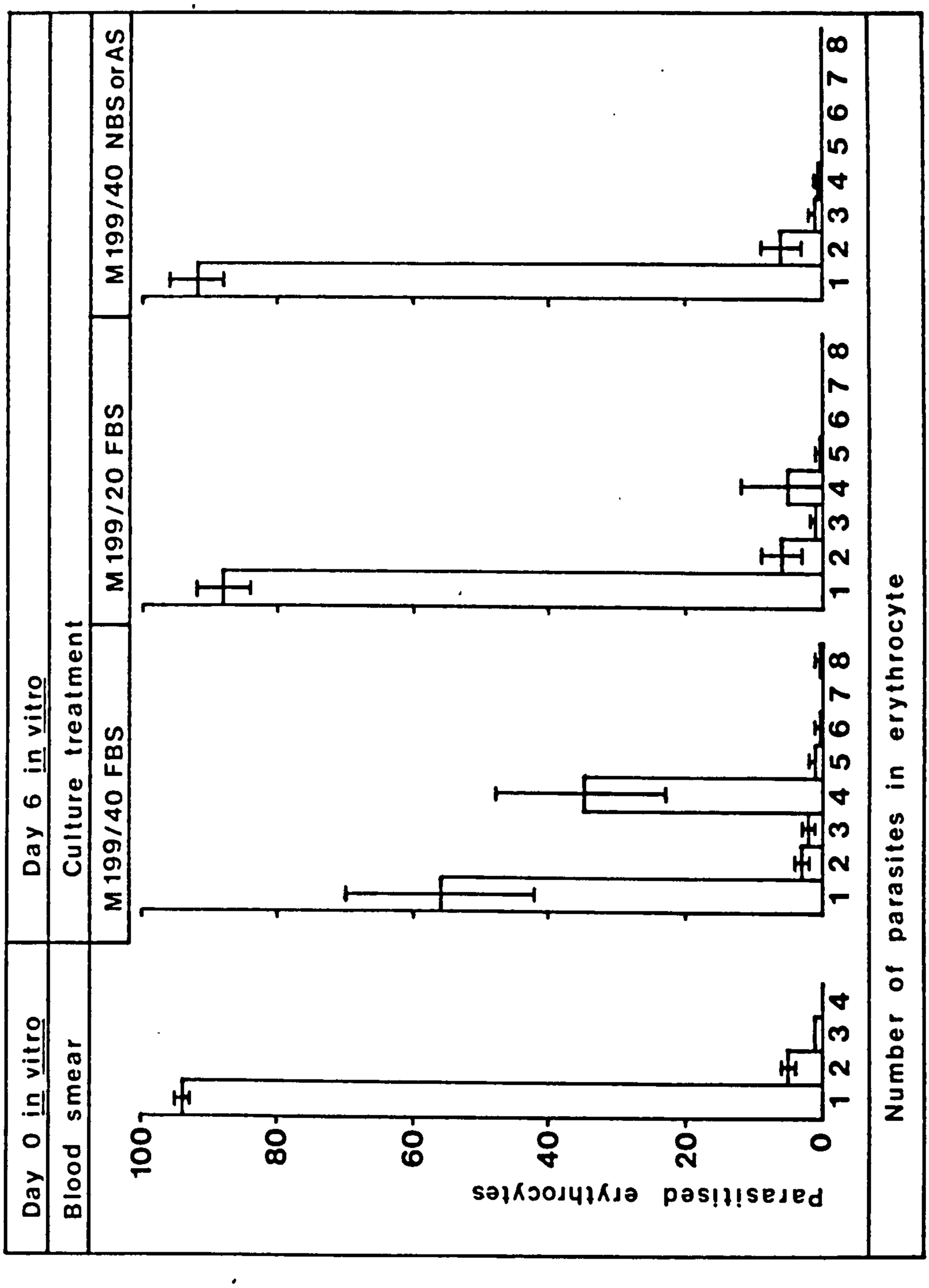

Day 0 in vitro
Blood smear
Day 6 in vitro
Culture treatment
M199/40 FBS
M199/20 FBS
M199/40 NBS or AS
Parasitised erythrocytes
100
80
60
40
20
0
1 2 3 4
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5
Number of parasites in erythrocyte
2
6
6
6
Number of samples

Figure 3.9 Theileria annulata (Ankara) cultures established with blood from calf 156 in experiment 3.6. Figures represent mean number of parasitized erythrocytes per 100 counted with specified number of parasites inside.


Day 0 in vitro
Blood smear
Day 6 in vitro
Culture treatment
M199/40 FBS
M199/20 FBS
M199/40 NBS or AS
Parasitised erythrocytes
100
80
60
40
20
0
1 2 3 4
1 2 3 4 5 6 7 8
Number of parasites in erythrocyte
Number of samples
2
6
6
6

increase in the number of intraerythrocytic parasites was greater in the cultures with FBS, regardless of whether the serum was heat inactivated, than in the cultures with normal bovine or autologous sera (Figures 3.8 and 3.9, Appendix 9). The interaction between factors was not significant in this analysis of variance.

3.7 Discussion

The first question to consider when evaluating the results of each experiment in this chapter was whether piroplasms multiplied in the stationary erythrocyte cultures established with T. annulata-infected blood. T. annulata was expected to divide and the daughter parasites to invade other erythrocytes. Therefore, an increase in the parasitaemia was the criterion originally selected for assessing multiplication.

There was no apparent increase in the parasitaemias to indicate that parasites had multiplied in experiment 3.2. The reduced oxygen tensions, depth of medium and depth of the erythrocyte layer may have inhibited diffusion of an oxygen concentration to the level of the parasites, sufficient to maintain growth. The concomitant 10% CO_2 tensions were perhaps excessive, although tensions of 8% CO_2 have been routinely administered to P. falciparum cultures with reduced O_2 tensions (Siddiqui and Palmer, 1981). In retrospect, the evaluation system might also have prevented the detection of any minor increase in the number of parasites during cultivation.

The evaluation system was altered after realising in experiment 3.3 that although the parasitaemia did not change, the number of parasites within each parasitized erythrocyte increased during the

cultivation period. Counts were subsequently made of the number of parasites in the erythrocytes.

The appearance of clusters of four small intraerythrocytic parasites in various configurations, including cross forms (Figure 3.1) was the most remarkable and consistent change observed in experiments 3.3-3.6. Intraerythrocytic quadruplet forms appeared to have resulted from the division of single piroplasms in cultures established with blood from cattle infected with either the Hissar (Experiments 3.3-3.5) or Ankara (Experiment 3.6) strains of T. annulata.

Intraerythrocytic division into four has been considered a characteristic feature of Theilera (Bettencourt et al., 1907). The morphologic appearance of T. annulata in vitro (Figure 3.2) was strikingly similar to the forms described by Dschunkowsky (1927) as developmental stages in the schizogonous multiplication of T. annulata in the erythrocytes of infected cattle. Dschunkowsky (1927, 1952) contended that schizogony generally resulted in four anaplasmoid daughter parasites but occasionally two or three minute parasites were seen in T. annulata-infected erythrocytes.

The incidence of multi-parasitized erythrocytes generally increases as the parasitaemia rises in T. annulata-infected cattle due, particularly during the initial stages, to the invasion of erythrocytes by the merozoites formed from microschizonts, as shown in the diagram in Figure 1.1. A single intralymphocytic microschizont produces hundreds of merozoites but the probability of an erythrocyte being invaded is initially low. Therefore, the majority of the parasitized erythrocytes in the low parasitaemia blood, used in these experiments, contained single piroplasms with a small proportion

containing two or three. This distribution of piroplasms in T. annulata-infected cattle has been interpreted as evidence that division occurs by binary fission (Levine, 1973; Soulsby, 1982). Quadruplet forms are generally seen in less than 5% of the parasitized erythrocytes in T. annulata-infected cattle (Sergent et al., 1945; Srivastava and Sharma, 1976a).

The morphology and number of intraerythrocytic parasites were carefully observed in each experiment in an attempt to determine the mode of parasite multiplication. An increase in the incidence of parasitized erythrocytes with two, three or more than four parasites was observed in T. annulata cultures but this change was minor compared to the consistent increase in the number of quadruplet forms.

Intraerythrocytic division appeared to result in a maximum of four parasites. Two observations support this contention. Firstly, the number of erythrocytes with more than four parasites after cultivation was never greater than the number of erythrocytes with two or three piroplasms in the blood used to establish cultures. Secondly, clusters of four small parasites were always seen within these multiparasitized erythrocytes (Figure 3.5).

Most of the small parasites seen in the samples from piroplasm cultures were in clusters, but occasionally some parasites had dispersed throughout the host erythrocyte. During the 6-8 day incubation period the number of parasitized erythrocytes did not increase significantly in the cultures. Parasites did not appear to be leaving the host erythrocytes and invading other erythrocytes in appreciable numbers. The increased incidence of three small parasites in an erythrocyte which corresponded to a decrease in the incidence of quadruplet

forms after day 6 *in vitro* suggested, however, that single parasites may have escaped after division into four (Figures 3.3 and 3.4).

The culture environment was not adequate to support continuous parasite propagation. After day 6-10 *in vitro* parasites became progressively more pycnotic and the cultures were discarded.

Different culture factors were tested in an attempt to facilitate parasite multiplication and erythrocyte invasion. Since the latter apparently did not occur *in vitro*, the increase in the total number of intraerythrocytic parasites per 100 parasitized erythrocytes after six days *in vitro* was used as the basis for comparing the different factors tested. The greatest parasite increases were in cultures with M199 and MEM-Alpha media (Experiments 3.3 and 3.4). The differences between the media and medium supplements tested, as determined by the increase in intraerythrocytic parasites between days 0 and 6, were either not statistically significant (Experiments 3.4 and 3.6) or only significant in cultures from one of the donor calves (Experiments 3.3 and 3.5). This discrepancy in the cultures established with blood from different calves suggested that the parasite's nutrient requirements may be influenced by the host erythrocyte.

Foetal bovine serum had an obvious stimulatory effect on parasite multiplication as compared to autologous and normal bovine sera (Experiments 3.3, 3.5 and 3.6). The quantitative variation in the chemical composition of FBS makes a comparison between sera difficult (Olmsted, 1967; Bittles, 1974; Honn, Singley and Chavin, 1975; Fox and Sanford, 1975). Generally, FBS is recognised as having greater growth-promoting properties than sera from older cattle (Ryley and Wilson, 1978). Hence FBS is added to the growth media when cultivating

the intralymphocytic stages of fastidious theilerial parasites (Brown, 1979).

In experiment 3.5 cultures grown in erythrocytes from calf 145 in medium with 20% FBS with and without supplements showed an increase in parasite numbers comparable to that seen in cultures grown with 40% FBS (Figure 3.6). Cultures from calf 146 with medium containing 20% FBS with hypoxanthine and glutathione supplements, grown on BAE monolayers, had parasite counts similar to those seen in cultures with 40% FBS in the complete medium (Figure 3.7). Parasites in cultures with BAE monolayers and M199/20 FBS supplemented with hypoxanthine only did not show comparable multiplication to that seen when glutathione was also present.

A unique characteristic of FBS is the high level of protein-glutathione mixed disulfides as compared to adult bovine and calf sera (Bump and Reed, 1977; Hoffeld and Oppenheim, 1980). Reduced glutathione (GSH) plays an essential role in protecting sulfhydryl groups in erythrocytes by acting as a reducing agent (Beutler, 1975; Grimes, 1980). Similarly, GSH may act by scavenging free radicals (Kosower and Kosower, 1978; Jakoby, 1978) formed at relatively high O_2 tensions *in vitro* (Halliwell, 1978). GSH appeared to be beneficial to *T. annulata* piroplasms (Experiments 3.5 and 3.6) but the stimulatory effect of FBS on parasite multiplication could not be attributed entirely to this compound. Supplementation of M199/20 FBS with GSH in experiment 3.6 did not result in parasite increases as great as those seen in M199/40 FBS.

Parasites in cultures with medium containing normal adult bovine or autologous sera retained their normal morphology but only a small

proportion of the parasites multiplied (Experiments 3.3 and 3.6). Essential nutrients or stimulatory factors present in FBS appeared to be deficient in these sera. Extensive counts indicated that parasites were not leaving the host erythrocyte after division and reinvading other cells. The possibility did, however, exist that a low incidence of reinfection might escape detection in periodic examinations of culture samples. An invasion assay was utilized in Chapter 4 to ascertain if reinvasion was occurring in T. annulata cultures with either FBS or NBS in the complete medium.

Further studies were conducted to evaluate the mode of multiplication of T. annulata by transmission electron microscopy in Chapter 5 and by in vivo experiments with infected cattle in Chapter 6. The behaviour of Theileria parva and Babesia bovis, respectively, were studied in experiments described in Chapters 7 and 8.

CHAPTER FOUR

EVALUATION OF ERYTHROCYTE INVASION BY THEILERIA ANNULATA IN VITRO

4.1 Introduction

Counts made from culture samples during the experiments in Chapter 3 indicated that parasites were not invading erythrocytes in vitro. The possibility existed that a low level of invasion might have occurred and not been detected by routine counting procedures. This might explain the observed behaviour of Theileria annulata in medium containing normal homologous (NBS) or autologous serum where quadruplet forms were never as numerous as in the corresponding cultures with foetal bovine serum (FBS). The observation seemed anomalous, as piroplasms might be expected to grow better in conditions closer to the in vivo environment than those provided by FBS.

A simple assay developed to test the infectivity of Plasmodium falciparum merozoites in vitro by adding fluorescein-stained, uninfected erythrocytes (Lamont, Saul and Kidson, 1981) was modified for use in T. annulata cultures. The modified method was initially tested in continuous cultures of Babesia bovis in which parasites do invade erythrocytes.

The invasion of erythrocytes by T. annulata in vitro was evaluated in cultures with medium containing either FBS or NBS.

4.2 Materials and Methods

The method employed for the invasion assay was as described in Section 2.9. Blood was collected from calves 194 and 195 on day 10

post-infection with T. annulata (Ankara) and used to prepare the 10% (v/v) concentrated erythrocyte suspensions in M199/40 FBS and M199/40 NBS.

B. bovis (Mexico) cultures were used as a positive control in this experiment. Details regarding the establishment of these cultures are given in Chapter 8. An aliquot of B. bovis (Mexico) culture suspension was mixed 1:4 with a suspension of fluorescein-stained erythrocytes in M199/40 NBS, to reduce the parasitaemia to 1-2%, and used to establish cultures.

Cultures were evaluated at 24 hour intervals as described in Section 2.9.

4.3 Results

The mean values of parasite counts made on days 0 and 6 from cultures with erythrocytes from the T. annulata-infected calves are displayed in Tables 4.1 and 4.2. Counts of the samples taken from cultures with fluorescein-stained erythrocytes were similar to the corresponding cultures with unstained erythrocytes.

Intraerythrocytic quadruplet forms were not observed in the parasitized erythrocyte suspensions on day 0 but appeared in all of the cultures between days 2 and 6. The highest incidence of erythrocytes with four parasites occurred in cultures with medium containing FBS. More than 25% and 70% of the parasitized erythrocytes in the FBS cultures of calves 194 and 195 respectively contained quadruplet forms by day 6.

The number of parasitized erythrocytes did not change appreciably in any of the cultures throughout the six day observation period.

Table 4.1 Theileria annulata (Ankara) cultures established with blood from calf 194: Diluted 1:2 with suspensions of unstained (US) or fluorescein-stained (FS) bovine erythrocytes.

Mean counts of 100 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside.

Day in vitro	Culture sampled	Number of parasites in erythrocytes									PRBC per 1000 erythrocytes
		1	2	3	4	5	6	7	8	12	
0	Pooled FS + US	96	3	1	0	0	0	0	0	0	12 ± 4
	Medium with FBS:										
	FS	70.5	2.2	0.2	25.2	1	0.7	0	0.2	0	8 ± 1
	US	67	2	0.5	29.8	0.5	0	0	0.2	0	10 ± 1
6	Medium with NBS:										
	FS	96.5	3	0	0.5	0	0	0	0	0	12 ± 2
	US	97.2	1.8	0.2	0.8	0	0	0	0	0	8 ± 2

Figures are mean counts based on parasites in 200 PRBC and mean ± SD per 1000 PRBC in 4 culture inocula day 0 and duplicate cultures sampled day 6.

Table 4.2 Theileria annulata (Ankara) cultures established with blood from calf 195: Diluted 1:2 with suspensions of unstained (US) or fluorescein-stained (FS) bovine erythrocyes.

Mean counts of 100 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside.

Day in vitro	Culture sampled	Number of parasites in erythrocytes									PRBC per 1000 erythrocytes
		1	2	3	4	5	6	7	8	12	
0	Pooled FS + US	97	2	1	0	0	0	0	0	0	19 ± 2
	Medium with FBS:										
	FS	26.8	0	0	70.8	1.2	0	0	1	0.2	20 ± 4
	US	26	0	1	70.8	1	0	0	1	0.2	18 ± 5
6	Medium with NBS:										
	FS	90	4	1.5	4.5	0	0	0	0	0	22 ± 2
	US	91.5	4.5	0.2	3.8	0	0	0	0	0	21 ± 1

Figures are mean counts based on parasites in 200 PRBC and mean ± SD per 1000 PRBC in 4 culture inocula day 0 and duplicate cultures sampled day 6.

In the invasion assay T. annulata, stained orange with ethidium bromide, were only seen in unstained erythrocytes (Figure 4.1). By comparison, after 24 hours, 50-60% of the fluorescein-stained erythrocytes in B. bovis cultures contained parasites (Figure 4.2) and the total number of parasitized erythrocytes had increased three-fold.

4.4 Discussion

Configurations of four small parasites, identical to the quadruplet forms seen in the experiments described in Chapter 3, appeared in all of the T. annulata cultures. The greatest increase in the incidence of erythrocytes with four parasites occurred in cultures with medium containing FBS (Tables 4.1 and 4.2).

The invasion assay employed in this experiment confirmed that parasites were not invading the normal bovine erythrocytes added to the T. annulata cultures (Figure 4.1). Parasite counts from cultures diluted with either unstained or fluorescein-stained erythrocytes were similar, thus indicating that the stain was not deleterious to the parasites.

The possibility that parasites invaded unstained but not stained erythrocytes seemed unlikely based on the daily counts of parasitized erythrocytes and comparisons with the B.bovis controls. B. bovis merozoites readily invaded fluorescein-stained erythrocytes (Figure 4.2). The parasite counts in B. bovis cultures diluted with stained erythrocytes were similar to the counts from cultures receiving unstained erythrocytes in preliminary experiments (P.A. Conrad, unpublished observations).

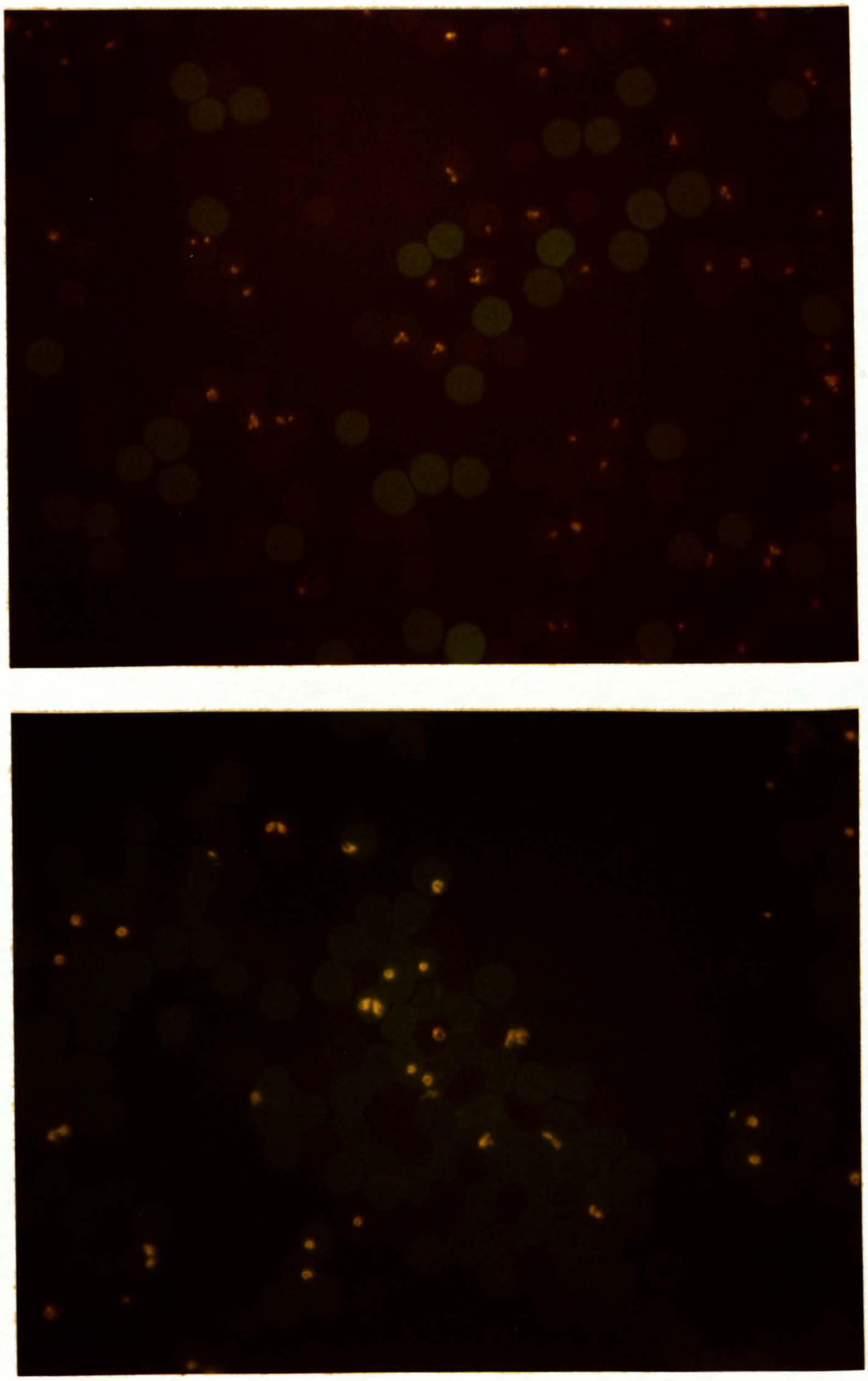
Figure 4.1 <u>Theileria annulata</u> (Ankara) after 6 days in stationary erythrocyte cultures established with blood from calf 195 and diluted with fluorescein-stained normal bovine erythrocytes.

(x 864)

Note the parasites, stained orange with ethidium bromide, are only in the unstained cells of calf 195.

Figure 4.2 After 24 hours in stationary erythrocyte cultures <u>Babesia bovis</u> (Mexico) has invaded and begun to divide within the fluorescein-stained normal bovine erythrocytes that were added to the cultures.

(x 864)



The higher incidence of quadruplet forms in cultures with medium containing FBS, as opposed to NBS, may be due to additional nutrients or stimulatory factors in the FBS. The general ability of FBS to act as a pH buffer (Barnes and Sato, 1980), promote cellular multiplication (Temin, Pierson and Dulak, 1972; Ham and McKeehan, 1979), scavenge deleterious oxygen-derived free radicals (Jakoby, 1978; Hoffeld and Oppenheim, 1980) and bind components, including metals, present at toxic levels in the medium (Vogt, Mischell and Dutton, 1969; Galembeck and Cann, 1974; Iscove and Melchers, 1978; Guilbert and Iscove, 1976) may have augmented parasite multiplication.

CHAPTER FIVE

EVALUATION OF THE INTRAERYTHROCYTIC MULTIPLICATION OF THEILERIA ANNULATA BY ELECTRON MICROSCOPY

5.1 Introduction

The development of single T. annulata piroplasms into four small parasites in stationary erythrocyte cultures appeared to represent a form of multiplication. Transmission electron microscopic studies described in this chapter were conducted to determine the ultrastructural features of the parasites in quadruplet forms and the mode of intraerythrocytic multiplication.

Since the advent of electron microscopy various developmental stages of protozoal parasites have been characterised. Based on similarities in their fine structure, many of the intracellular protozoa appear to feed during a trophozoite stage before undergoing nuclear and cytoplasmic division as schizonts to form uninucleate merozoites,as reviewed by Rudzinska and Vickerman (1968), Ladda (1969), Aikawa (1971), Aikawa and Sterling (1974), Scholtyseck (1979) and Rudzinska (1981). Trophozoite, schizont and merozoite stages have been identified in the intralymphocytic development of Theileria (Jarrett and Brocklesby, 1966; Büttner, 1967b; Mugera and Munyua, 1973; Schein, Mehlhorn and Warnecke, 1978; Jura et al., 1983) and Babesia equi (Moltmann et al., 1983a). Trophozites and merozoites were recognized as intraerythrocytic developmental stages of Babesia spp. (Kreier, Graveley, Seed, Smucker and Pfister, 1975; Rudzinska, Trager, Lewengrub and Gubert, 1976; Friedhoff and Scholtyseck, 1977; Potgieter and Els, 1977, 1979; Todorovic, Wagner and Kopf, 1981).

The terms merozoite and trophozoite have not, however, been used to distinguish the intraerythrocytic forms of Theileria which are simply referred to as piroplasms (Theiler, 1904; Nuttall, 1914; Cowdry and Danks, 1933; Barnett, 1977). Multiplication by binary fission was observed in electron microscopic studies of T. annulata (Schein et al., 1977) and T. mutans (Büttner, 1966) but the apical complex structures, characteristic of merozoites, were not described.

The basis for many of the terms used to describe the ultrastructure of parasites is often obscure. The newcomer to electron microscopy must rely on making comparisons between the structures observed and those described in previous studies, preferably of closely related parasites. The terminology applied in the presentation of results obtained from the transmission electron microscopic (TEM) studies of T. annulata, described in this chapter, was decided upon after a review of the relevant literature and discussions with parasitologists experienced in protozoal ultrastructure (D.W. Brocklesby, L.H. Bannister and K. Vickerman, personal communications).

Electron micrographs of intralymphocytic merozoites and typical piroplasms, in samples from T. annulata-infected cattle, were included in this study to serve as a comparison with the developmental forms observed in culture samples.

5.2 Materials and Methods

The protocol for sample fixation and section preparation was as described in Section 2.12.2. Giemsa stained cytocentrifuge smears were prepared from aliquots of each sample processed for TEM.

The parasitized samples were derived from two T. annulata-infected calves:

(a) Calf 153 infected with T. annulata (Ankara) - A biopsy sample was taken from the right prescapular lymph node on day 13 post infection with a 14 gauge 1½ inch needle and expelled directly into a solution of 2.5% glutaraldehyde fixative. Blood was collected on day 14 post infection and defibrinated. Aliquots were either fixed as a pellet for TEM or used to prepare a 3% (v/v) suspension of concentrated erythrocytes, obtained as described in Section 2.8.2, in complete medium consisting of 60% M199 and 40% FBS. Stationary erythrocyte cultures were established with 5 ml aliquots of the 3% culture suspension in vertical flasks, gassed with 5% CO_2 in air and maintained as described in Section 2.8.3. Samples for TEM were taken on day 2 in vitro.

(b) Calf 148 infected with T. annulata (Hissar) - Stationary erythrocyte cultures were established in vertical flasks with 5 ml aliquots of a 3% (v/v) concentrated erythrocyte suspension in complete medium prepared using blood obtained on day 14 post infection, as described in Section 2.8.2. The complete medium consisted of 80% MEM Alpha medium with 25mM HEPES buffer and 20% FBS, supplemented with 2 mg glucose, 50 μg hypoxanthine and 50 μg reduced glutathione per ml of the final preparation. Cultures were gassed with 5% CO_2 in air and maintained as described in Section 2.8.3. Samples for TEM were taken on day 4 in vitro.

5.3 Results

5.3.1 T. annulata in lymph node and blood samples: The ultrastructural features of the exoerythrocytic (intralymphocytic) merozoites of T. annulata are shown in the electron micrograph in Figure 5.1 taken of a lymph node biopsy sample. Differential merozoites in an infected lymphoid cell each had a single, limiting plasmalemmal membrane, a large nucleus with homogeneous karyoplasm, and a double-membraned organelle (mitochondrion?). Distinctive structures, consisting of an inner membrane segment, rhoptries and micronemes, constituted the apical complex of the merozoite.

Counts of intraerythrocytic T. annulata piroplasms in blood and culture samples are shown in Table 5.1. Typical theilerial piroplasms as seen in Figures 5.2 and 5.3 ranged in size from 0.9-1.5μ in length and 0.6-1.0μ in width. Piroplasms were situated directly in the cytoplasm of the host erythrocyte, without a parasitophorous vacuole. Piroplasms had a single, limiting plasmalemmal membrane, cytoplasm with numerous free ribosomes and a prominent nucleus (0.7-0.9μ x 0.3-0.8μ) bordered by a double membrane. The nucleoplasm was homogeneously composed of fine granules and fibrils without a nucleolus. Cytoplasmic organelles consisted of two double membraned structures, (0.3-0.6μ x 0.1-0.4μ) located at the end opposite to the nucleus, which had electron-lucent centres with diffuse filamentous strands radiating into the lumen from the inner membrane (Figure 5.3). A cytostome and food vacuole were seen which contained material similar in appearance to the cytoplasm of the erythrocyte. Apical complex structures, seen in exoerythrocytic merozoites, were not observed in typical piroplasms.

Figure 5.1 Section through a lymphoid cell (HC) infected with Theileria annulata (Ankara). Differentiated merozoites (ME) possess nuclei (N), double membraned organelles (M) and apical complex structures, consisting of segments of inner membranes (IM), rhoptries (R) and micronemes (MC).
Bar = 0.5μ (x 41250)


HC
ME
IM
MC
N
M
R
N
ME
HN

Table 5.1 Counts of Theileria annulata in blood and stationary erythrocyte culture samples infected with the Ankara and Hissar strains

Sample	Day in vitro	Number of parasites in erythrocytes 1	2	3	4	5	6	PRBC per 1000 erythrocytes
Ankara strain - Calf 153:								
Fresh blood*	0	68	27	3	2	0	0	475
Defibrinated blood**	0	68	24	6	2	0	0	483
Culture suspension	2	58	26	4	8	3	1	490
Hissar strain - Calf 148:								
Fresh blood*	0	90	8.5	1	0.5	0	0	127
Defibrinated blood**	0	89	10.5	0.5	0	0	0	130
Culture suspension	4	62.5	9	7	21	0.25	0.25	126

*Fresh blood smears prepared at time of blood collection.

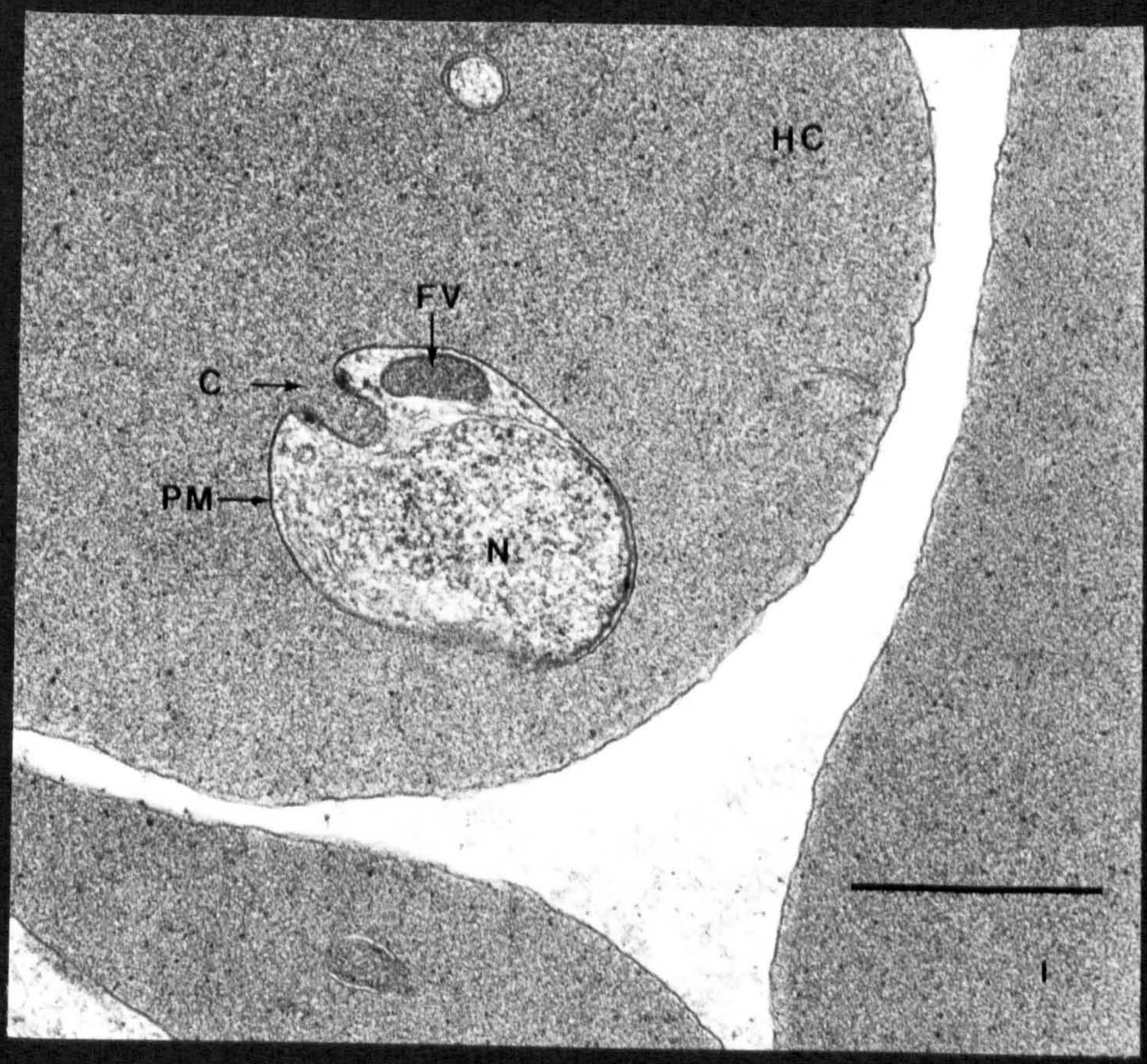
**Defibrinated blood smears prepared at time of fixation, 30-60 minutes after collection.

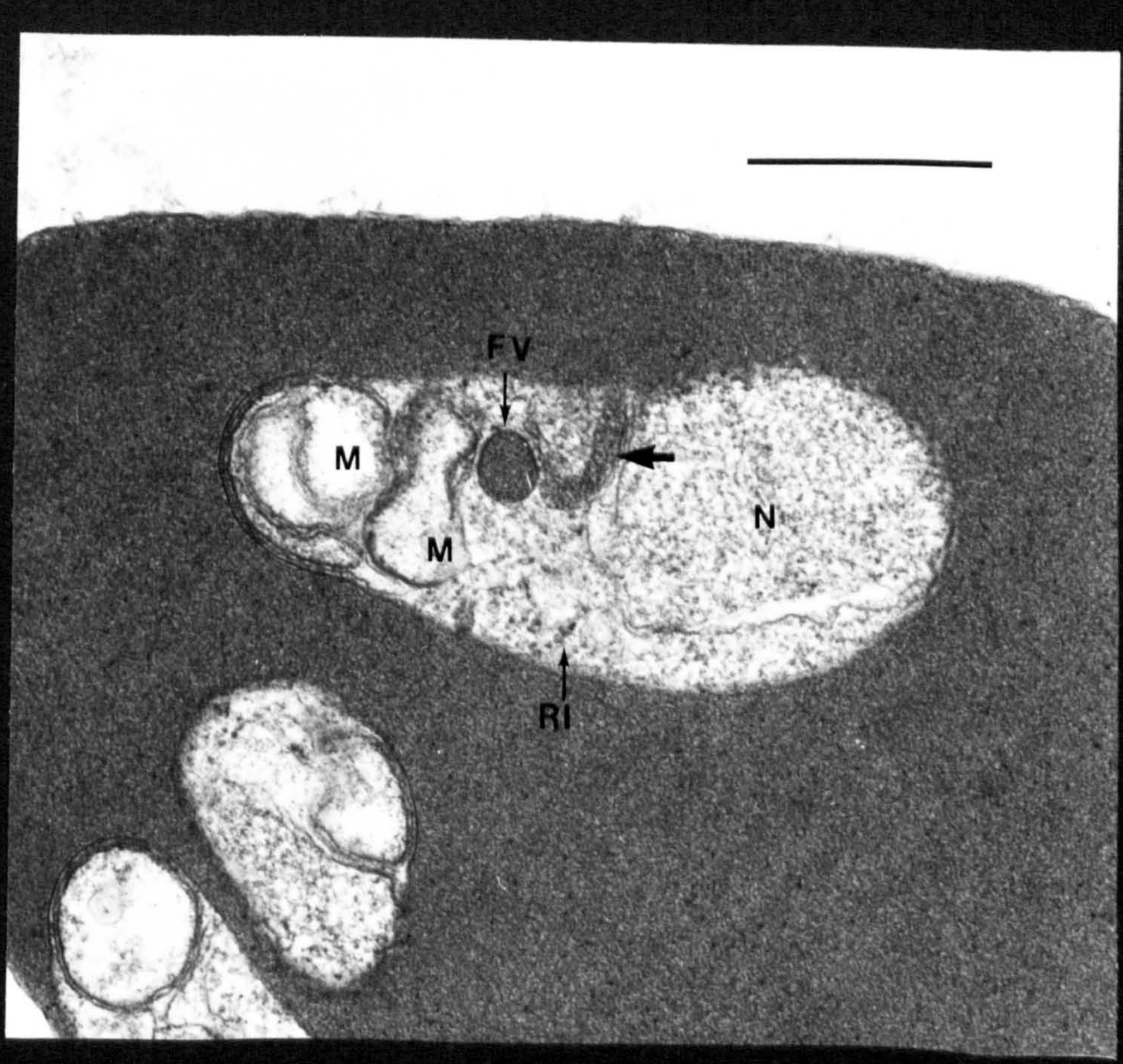
Values are mean counts of parasites in 200 parasitized erythrocytes (PRBC) and PRBC per 1000 erythrocytes in 2-6 replicates for each sample.

Typical piroplasms in erythrocytes (HC) taken
from a calf infected with Theileria annulata (Ankara)

Figure 5.2 Note the single plasmalemmal membrane (PM), prominent nucleus (N), vacuole filled with erythrocyte cytoplasm (FV) and cytostome (C).
Bar = 0.5μ (x 53250)

Figure 5.3 Vacuolated double membraned organelles (M) with diffuse filamentous invaginations, are situated at the opposite pole from the large nucleus (N). The electron dense structure (←) extending to the food vacuole (FV) may represent a transected cytostome. Numerous ribosomes (RI) are free in the cytoplasm.
Bar = 0.5μ


HC
FV
C
PM
N
I


FV
M
M
N
RI

5.3.2 T. annulata in stationary erythrocyte culture samples: Many of the intraerythrocytic parasites seen in vitro resembled the typical piroplasms observed in the blood of T. annulata-infected cattle. Figure 5.4 shows a T. annulata (Ankara) piroplasm after two days in vitro. In addition to the ultrastructural features described in Section 5.3.1 for typical piroplasms, electron dense microtubular structures were occasionally seen in cultivated piroplasms (Figures 5.4 and 5.5).

Segments of double membrane, beneath the plasmalemma, and electron dense rhoptries (0.1-0.2μ) were observed in some parasites after two to four days in vitro (Figure 5.5). The appearance of these structures marked the site of formation of merozoites. One, to a maximum of four, merozoite anlagen were seen within a single plasmalemmal membrane (Figure 5.6). Small electron dense micronemes (10-40nm) and double membraned organelles (0.1-0.3μ x 0.08-0.2μ) were frequently associated with the apical structures of merozoite anlagen.

The nucleoplasm of dividing parasites was segmented into several nuclei (Figure 5.7). The nuclei remained centrally oriented as the merozoite anlagen formed protrusions of the plasmalemma in the areas where the segments of inner membranes and rhoptries appeared.

Intraerythrocytic merozoites were seen in clusters of two, three or four, often with their nuclei in close proximity (Figures 5.8-5.11). Merozoites were spherical or slightly pyriform with a diameter range of 0.5-0.7μ. The apical complex structures consisting of a double inner membrane, one to two rhoptries, and micronemes were present in the intraerythrocytic merozoites. The only other cytoplasmic structures observed in differentiated merozoites were single organelles bounded by a double membrane, and abundant free ribosomes.

Each intraerythrocytic merozoite had a prominent nucleus surrounded by a double membrane. The nuclear membrane was generally discernable even when the plasmalemma of the merozoite was continuous with the membrane of the residual body (Figure 5.11). The residual body appeared either as a vacuolated structure with diffuse osmiophilic filaments (Figures 5.10 and 5.11) or as a solid body with a variable proportion of homogeneous granular material resembling nucleoplasm (Figures 5.8 and 5.9).

5.4 Discussion

The purpose of this study was to determine the ultrastructural features of the intraerythrocytic forms of T. annulata which appeared to be multiplying in vitro. Electron micrographs of the exoerythrocytic merozoites and typical piroplasms in samples from T. annulata infected cattle were included for morphological comparisons.

In Giemsa stained smears merozoites that bud from intralymphocytic schizonts of T. annulata are identical in size and appearance to parasites in quadruplet forms. Electron micrographs revealed the same ultrastructural features, consisting of a nucleus, double membraned organelles, segments of inner membranes, rhoptries and micronemes, in both the intralymphocytic (Figure 5.1) and intraerythrocytic merozoites (Figures 5.8-5.11) of T. annulata

Schein et al. (1977) observed segments of inner membrane in comma-shaped T. annulata piroplasms which they propounded to be remnants of the invading merozoite's inner pellicular complex.

Figures 5.4-5.11 are of Theileria annulata
in stationary erythrocyte cultures.

Figure 5.4 T. annulata (Ankara) on day 2 in vitro showing the features of a typical piroplasm with a single plasmalemma (PM), a large nucleus (N), two double membraned organelles (M), a cytostome (C) and numerous free ribosomes (RI). A bundle of electron dense microtubules (MT) is associated with the outer nuclear membrane.
Bar = 0.5μ (x 53250)

Figure 5.5 T. annulata (Hissar) on day 4 in vitro. Note the segments of inner membranes (IM) with pairs of electron dense rhoptries (R) beneath the plasmalemma. The cytoplasm contains a double membraned vacuole (M) and a bundle of electron dense microtubules (MT).
Bar = 0.5μ (x 41250)


HC
M
MT
PM
C
RI
N

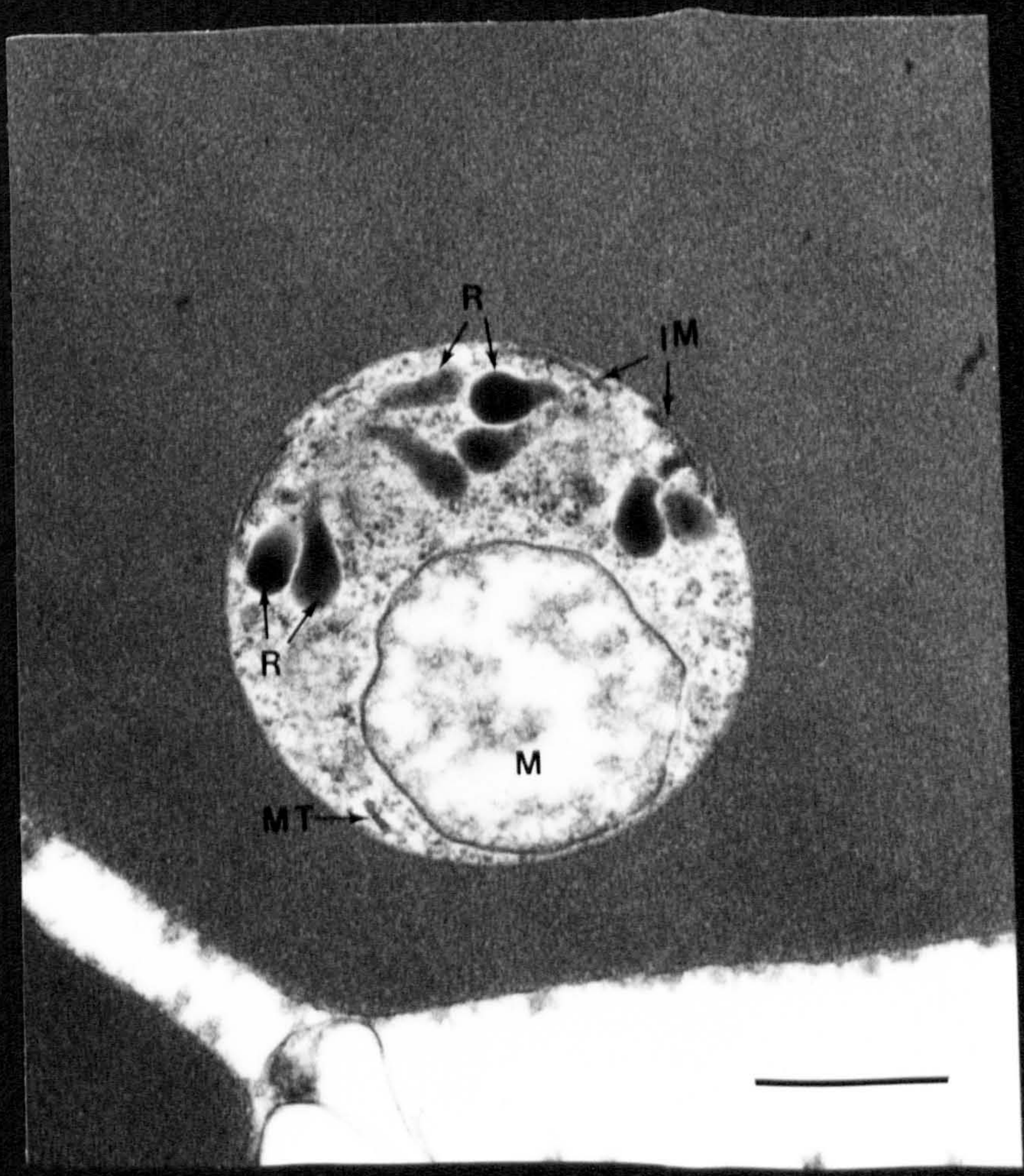
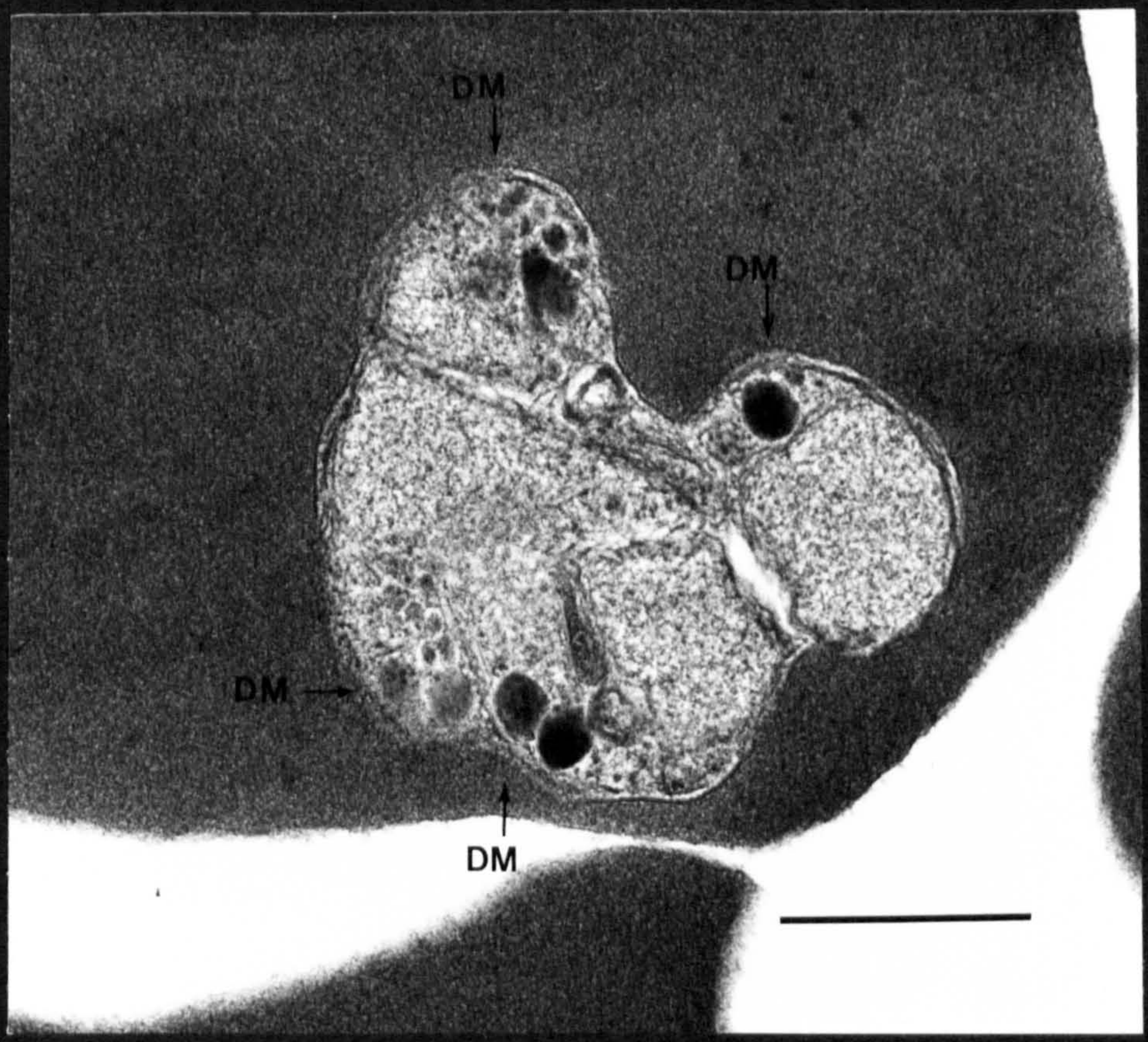

R
IM
R
M
MT

Figure 5.6 Section through T. annulata (Hissar) in vitro with four developing merozoites (DM) within a single limiting plasmalemmal membrane.
Bar = 0.5 μ (x 53250)

Figure 5.6(i) Diagrammatic representation of the merozoite anlagen (DM) in Figure 5.6 delineating the four individual nuclei (N), the inner membrane segments (IM), rhoptries (R), micronemes (MC) and double membraned organelles (M).


DM
DM
DM
DM

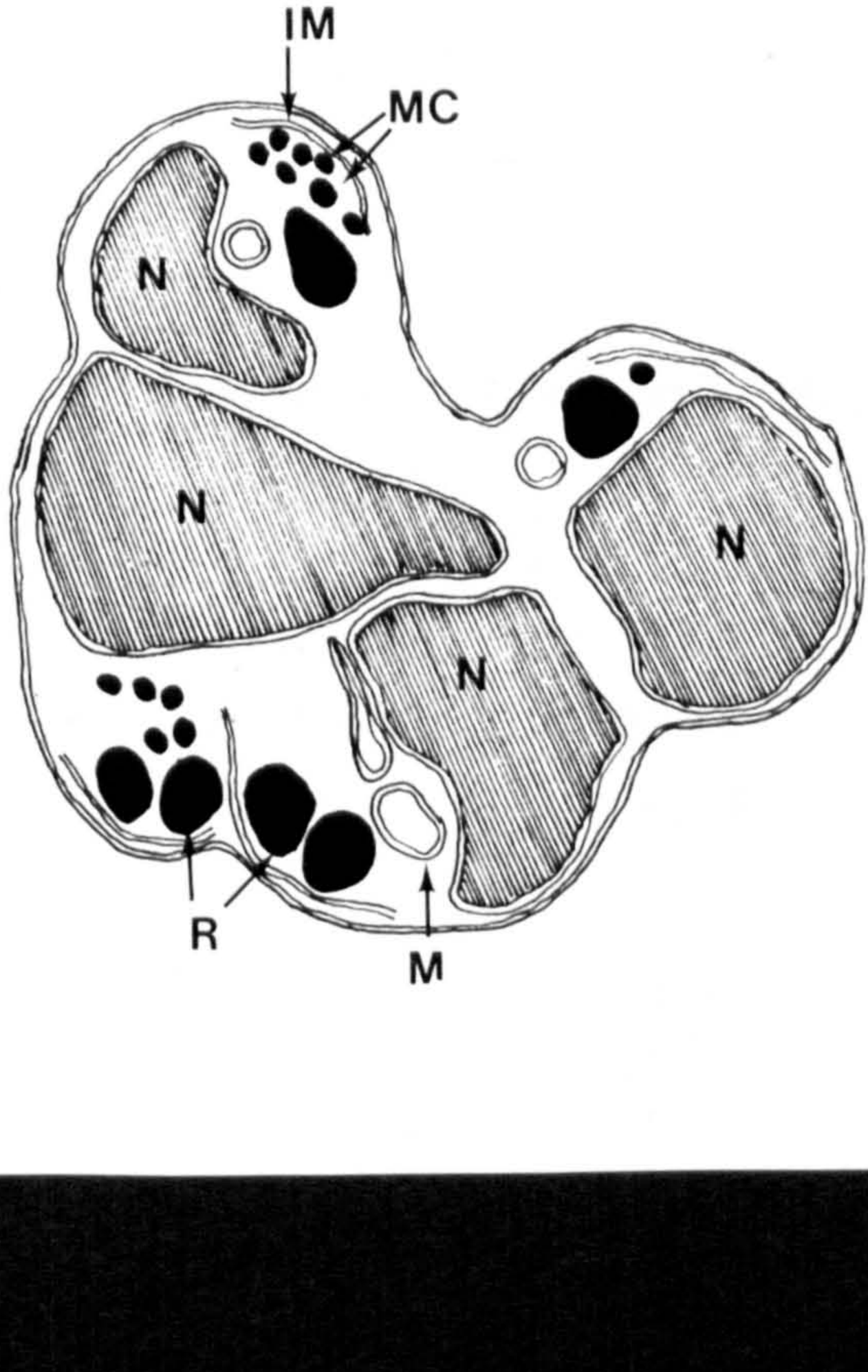

IM
MC
N
N
N
N
R
M

Figure 5.7 Section through T. annulata (Hissar) in vitro showing three merozoite anlagen with distinctly separate nuclei (N). The apical complex with rhoptries (R) and segments of inner membranes (IM) are apparent in two merozoite anlagen.

Bar = 0.5μ (x 87500)

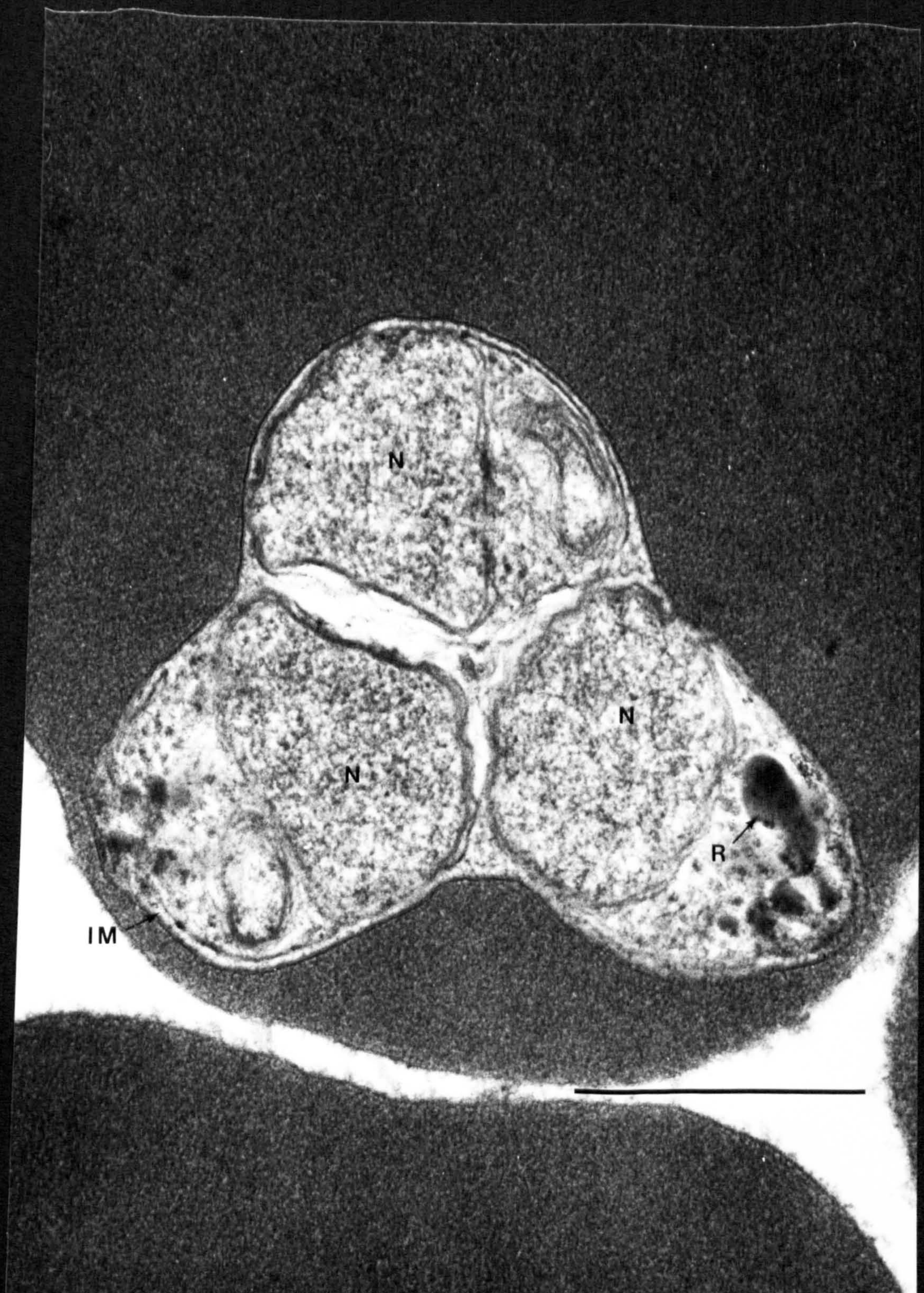

N
N
N
R
IM

Figure 5.8 T. annulata (Hissar) in vitro: four merozoites (ME) attached to a residual body (RB).
Bar = 0.5 μ (x 87500)

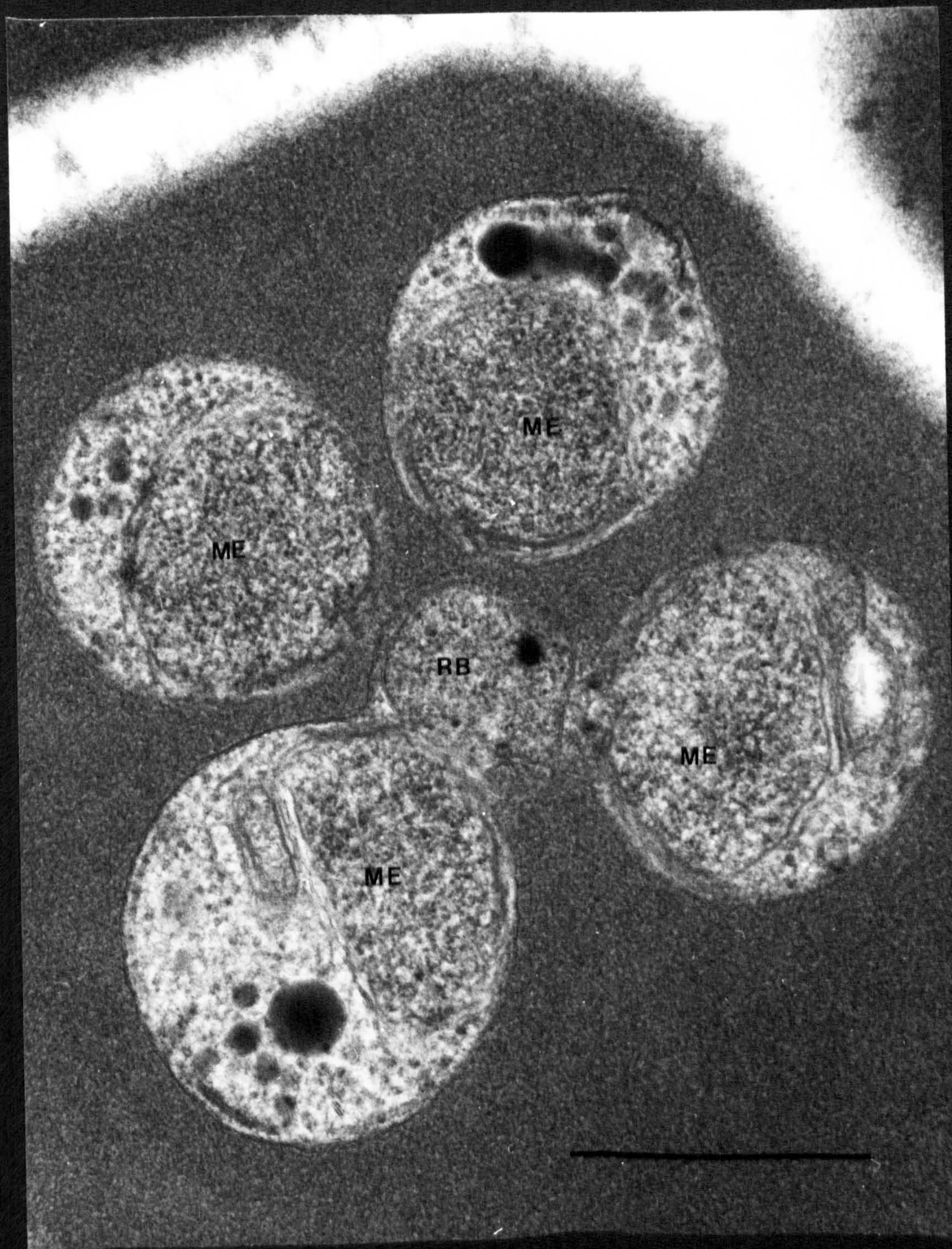

ME
ME
RB
ME
ME

Figure 5.9 Three merozoites of T. annulata (Ankara) in vitro attached to a residual body (RB). Note the apical segments of inner membranes (IM), rhoptries (R), micronemes (MC) and double membraned organelles (M).
Bar = 0.5μ (x 53250)

Figure 5.10 A pair of differentiated T. annulata (Hissar) merozoites (ME) in vitro.
Bar = 0.5 μ (x 53250)

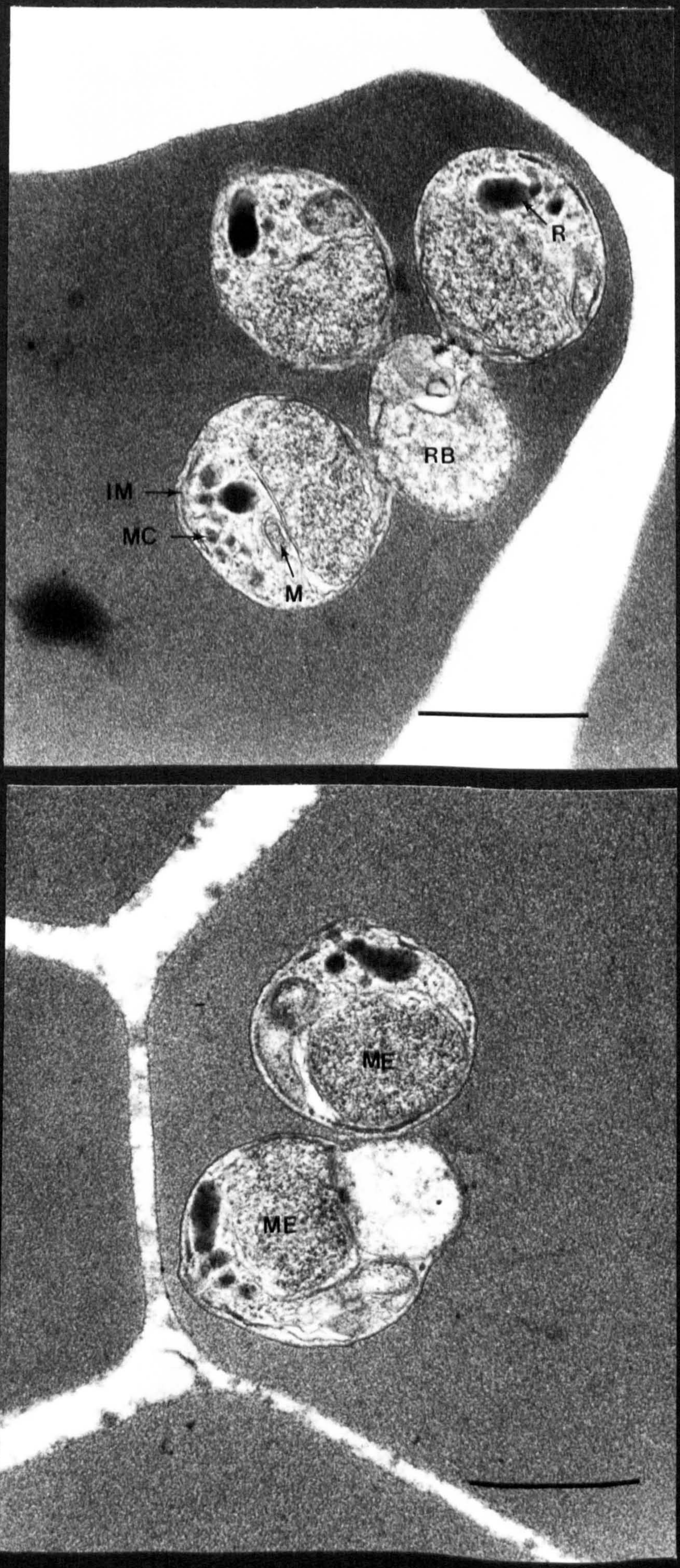
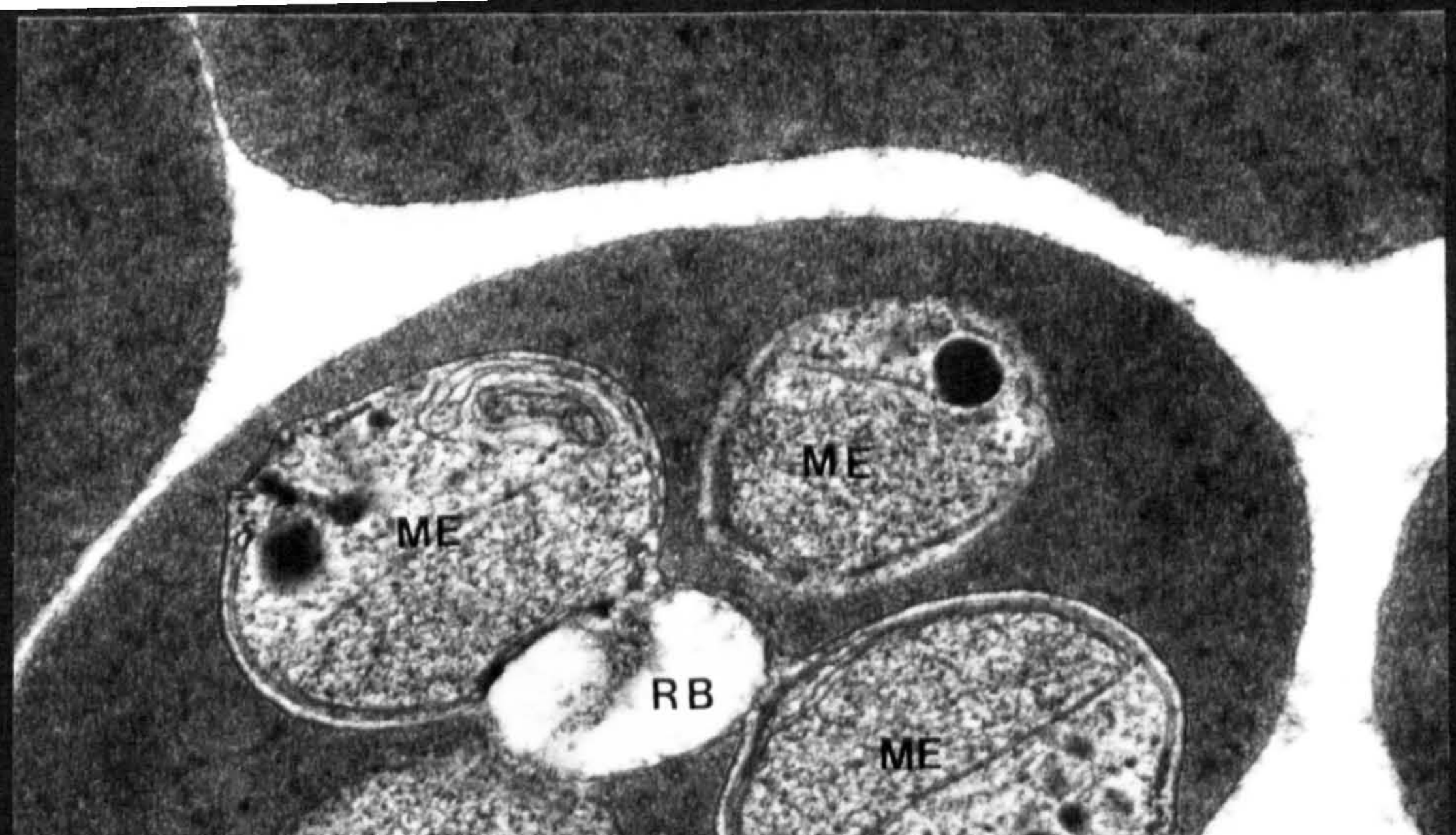
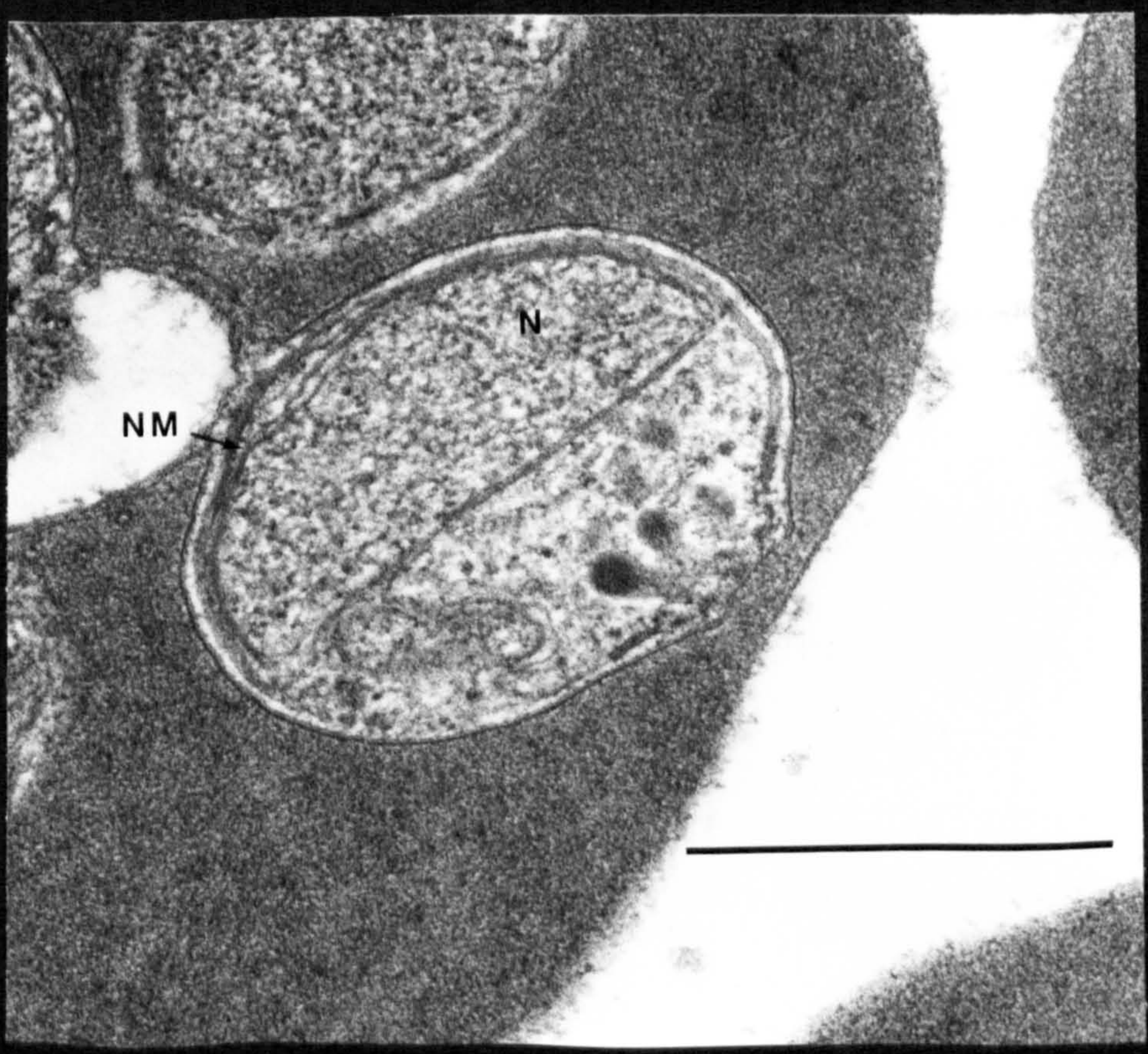

R
RB
IM
MC
M
ME
ME

Figure 5.11 Quadruplet formation of T. annulata (Ankara) merozoites (ME) attached to a residual body (RB).
Bar = 0.5 μ (x 53250)

Figure 5.11(i) Higher magnification of a merozoite in Figure 5.11. Note that the merozoite nucleus (N) is bounded by an intact double membrane (NM).
Bar = 0.5μ (x 90000)


ME
ME
RB
ME
ME


N
NM

The apical complex structures of theilerial merozoites are generally thought to disappear after erythrocyte invasion, as occurs in Plasmodium (Bannister, Butcher, Dennis and Mitchell, 1975) and Babesia (Rudzinska et al., 1976).

The predominant ultrastructural features of the typical piroplasms in defibrinated blood (Figures 5.2 and 5.3) and a proportion of the cultivated parasites (Figure 5.4) correspond to structures described in previous electron microscopic studies of the intraerythrocytic stages of Theileria (Büttner, 1966, 1967a; Schein et al., 1977). Defibrinated parasitized blood, fixed as a pellet within 30-60 minutes of collection, was used for comparisons with cultivated piroplasms after numerous attempts to fix fresh blood in suspension proved unsuccessful. Parasites in defibrinated blood were comparable in number and morphology to those seen by light microscopy in Giemsa stained fresh blood smears (Table 5.1).

Single-membraned vacuoles filled with erythrocyte cytoplasm, and the cylindrical, lateral plasmalemmal invagination referred to as a cytostome (Aikawa, Hepler, Huff and Sprinz, 1966) have been considered to be evidence of phagotrophy by Theileria (Büttner, 1966, 1967a) and other intraerythrocytic protozoa (Rudzinska and Trager, 1957, 1959; Aikawa and Jordan, 1968; Frerichs and Holbrook, 1974; Sénaud, Chobotar and Scholtyseck, 1976). The presence of these structures in typical T. annulata piroplasms suggests that they may be referred to a trophozoites (Greek "trophe" = nourishment; "zoion" = animal).

The term trophozoite is applied cautiously to describe this stage, since electron microscopic examination of fixed sections may implicate but certainly cannot prove that parasites are feeding.

Cytostomes without food vacuoles occur in exoerythrocytic stages of Theileria (Büttner, 1967a; Schein et al., 1978; Fawcett, Büscher and Doxsey, 1982) and some Plasmodium species (Hepler, Huff and Sprinz, 1966; Aikawa, Huff and Sprinz, 1968) where their function is uncertain. Structures that appeared to be food vacuoles in some parasites have proved to be intracellular cavities not associated with nutrition (Vivier and Petitprez, 1972) or mere invaginations of the host cytoplasm (Rudzinska, 1976). Further studies are required to fully elucidate the mechanism of nutrient acquisition by the intraerythrocytic stages of Theileria. The terminology as applied is, however, consistent with the usage of the word trophozoite to describe the growing, predivision stage of Babesia species (Rudzinska, 1976; 1981; Friedhoff and Scholtyseck, 1977; Potgieter and Els, 1977, 1979).

The appearance of segments of a double layered membrane and rhoptries beneath the plasmalemma of T. annulata indicated that merozoites had begun to form (Figure 5.5). The two layers of the inner membrane were distinguishable with optimal fixation and sectioning in a plane perpendicular to the membrane (Figure 5.8). Paired electron dense bodies, first noted in plasmodial sporozoites (Garnham, Bird, Baker and Bray, 1961), were given the name rhoptries by Scholtyseck and Mehlhorn (1970) and have since been recognized as characteristic organelles in the motile, infective stages of the Apicomplexa (Levine, 1970; Aikawa and Sterling, 1974; Scholtyseck, 1979). The smaller electron dense bodies within the apical complex of T. annulata merozoites had the appearance of micronemes which may be distinct organelles, rhoptry precursors, or merely fragments of transected rhoptries (Aikawa et al., 1966; Scholtyseck and Mehlhorn,

1970). Rhoptries and micronemes are supposed to contain lytic enzymes which facilitate cellular invasion by merozoites (Ladda, Aikawa and Sprinz, 1969; Bannister et al., 1975; Kilejian, 1976; Rudzinska et al., 1976; Aikawa, Miller, Johnson and Rabbege, 1978).

Double membraned organelles, similar to those seen in T. annulata merozoites, are thought to be acristate protozoal mitochondria (Aikawa, 1971; Rudzinska and Trager, 1976; Langreth, Jensen, Reese and Trager, 1978; Weber, 1978; Fawcett et al., 1982). Weber (1980, 1982) demonstrated the presence of the respiratory enzymes within the double membraned structures in T. annulata sporozoites. Thus, indirect evidence suggests that similar organelles in other parasite stages also function metabolically as mitochondria.

The specific mechanism of nuclear division was not detectable in electron micrographs of T. annulata. Microtubular structures were occasionally associated with the outer nuclear membrane (Figure 5.4) or seen in the cytoplasm (Figure 5.5) of the parasite, but their function was not known. Schein et al. (1977) described extranuclear microtubules in the comma forms of T. annulata; some of these forms divided by binary fission. Intranuclear microtubules suggestive of true mitotic organizing centres, as seen in Plasmodium (Aikawa and Seed, 1980; Bannister and Sinden, 1982) were not observed in this study.

Nuclear division appeared consistently to precede cytoplasmic division with several distinct nuclei seen within the limiting plasmalemma of a single T. annulata parasite (Figures 5.6 and 5.7). Careful examination may be required to discern the boundaries of the four merozoite anlagen in Figure 5.6. This particular electron

micrograph emphasises that, unlike samples prepared for light microscopy where parasites are flattened on a smear, the parasites in block samples processed for TEM retain their three dimensional arrangement. After sectioning, however, only the structures transected in one plane are clearly visible in the electron micrograph. This explains why one merozoite anlage in Figure 5.6 is only partially apparent, and less distinct than the other three. The same concept must be considered when interpreting the electron micrographs with two (Figure 5.10) or three (Figure 5.9) parasites apparently in the process of separation. Additional parasites may be closely associated but in a different plane of section.

The apical pole of intraerythrocytic merozoites in clusters projected towards the periphery while the nuclei remained centrally oriented, as seen in light micrographs of quadruplet forms (Figures 3.1 and 3.2). Nuclear division appeared, however, to be completed before the individual merozoites separated from the residual body (Figures 5.8-5.11).

Three basic modes of multiplication have been proposed for the intraerythrocytic Piroplasmida, namely binary fission, budding and schizogony. In both binary fission and schizogony, nuclear division must precede cytoplasmic division but in the latter more than two merozoites can be formed (Adam, Paul and Zaman, 1971; Levine, 1973; Aikawa and Sterling, 1974). The term budding has been used to describe the final separation of merozoites from the parent schizont. For example, in Figure 5.1 the exoerythrocytic merozoites of T. annulata were formed by budding from an intralymphocytic microschizont. The definition of budding, when considered as an

independent process of asexual reproduction, denotes, however, an unequal fragmentation of the nucleus and cytoplasm resulting in parasites markedly different in size (Wenyon, 1965; Soulsby, 1982).

There are divergent views on the application of these terms. Studies on B. microti and B. rodhaini, using electron microscopy and freeze etching, suggested that the division into "maltese" cross forms was by schizogony (Kreier et al., 1975). Rudzinska and Trager (1977) found that during division the nucleus of B. microti remained as a single body which extended into the 1-4 budding merozoites. The theory was put forth that Babesia divide within erythrocytes by budding, and not by either binary fission or schizogony, because nuclear division was not completed before cytoplasmic division (Rudzinska and Trager, 1977; Rudzinska, 1981). Interestingly, schizogony was recognised by the same author as the mode of intra-erythrocytic multplication for Plasmodium despite the fact that nuclear division in some species occurred simultaneously with cyto-plasmic division (Rudzinska and Vickerman, 1968; Rudzinska, 1969).

The division of Babesia and Theileria into two daughter parasites of equal size has been called binary fission (Büttner, 1966, 1968; Schein et al., 1977; Scholtyseck, 1979). Yet, by definition the same process may be referred to as schizogony. Potgieter and Els (1977, 1979) concluded that both B. bigemina and B. bovis divided by a form of intraerythrocytic schizogony into two daughter merozoites. Aikawa and Sterling (1974) proposed that the division of Babesia into two and Theileria into four merozoites was by a process of simple fission that resembled schizogony.

Consideration was given to these varied opinions. In conclusion the evaluations in this chapter showed that, in vitro, T. annulata divides by intraerythrocytic schizogony. Multiplication proceeds from the initial formation of the apical cytoplasmic structures of merozoite anlagen beneath the plasmalemma, to nuclear division and finally, the separation of a maximum of four differentiated merozoites from the residual body.

CHAPTER SIX

EVALUATION OF INTRAERYTHROCYTIC MULTIPLICATION IN CATTLE INFECTED WITH THEILERIA ANNULATA

6.1 Introduction

The purpose of the experiment described in this chapter was to evaluate the mode of intraerythrocytic multiplication of Theileria annulata in vivo. There is general agreement that T. annulata is capable of division in vivo and the following characteristic features of the infection have been considered as supportive evidence:

(a) During the course of tropical theileriosis numerous multi-parasitized erythrocytes are seen with piroplasms in configurations suggestive of division (Dschunkowsky, 1927; Schein et al., 1977).

(b) The disease can be transmitted by the inoculation of blood containing piroplasms (du Toit, 1930; Adler and Ellenbogen, 1935).

(c) Cattle infected with T. annulata remain chronic carriers, infective for ticks and subject to relapses when immunologically compromised, for example by severe stress or splenectomy (Sergent et al., 1931; Vélu, 1933; Sergent et al., 1945; Hooshmand-Rad, 1976; Srivastava and Sharma, 1976b).

The major obstacle in a study on the kinetics of piroplasm replication is the virtual impossibility, in natural infections, of distinguishing whether the piroplasms counted are the result of merozoites formed from schizonts within infected lymphoid cells or from merozoites produced by intraerythrocytic multiplication. This experiment was designed with the intention of isolating the intraerythrocytic cycle of multiplication by taking blood from a T. annulata (Ankara)

carrier calf and filtering the blood to remove any macroschizont-infected lymphoid cells. One calf was to receive the filtered blood, while a control calf would be inoculated with unfiltered blood. A comparison between the parasitic reactions in the two calves would serve as a means of assessing whether macroschizont-infected lymphoid cells had been present in the donor's blood.

The presence of macroschizonts in the donor and recipient calves would be monitored by the examination of blood and lymph node biopsy smears. In addition, weekly in vitro isolations of peripheral blood lymphocytes would give any cells infected with macroschizonts an opportunity to multiply in the cultures to a detectable level (Stagg, Brown, Crawford, Kanhai and Young, 1974). The ultimate test to ensure that the recipient calves had not received macroschizont-infected lymphoid cells was to observe their response to homologous challenge. Sporozoites derived from ticks which fed as nymphs on the donor calf would be used to prepare the challenge inocula. Neitz (1959, 1964) applied the same criteria in the transmission experiments that led him to conclude that piroplasms of Theileria mutans and T. parva could be maintained in the absence of schizonts.

If the piroplasms of T. annulata were isolated in vivo it might be possible to discern whether multiplication occurred by intraerythrocytic schizogony into four parasites, as observed in vitro, by binary fission, or by some different method.

6.2 Materials and Methods

6.2.1 Cattle:

(a) Carrier calves - Calves 154 and 155 were infected with a sporozoite stabilate of T. annulata (Ankara) on 11.2.82;

they recovered after treatment on 21.2.82 with the antitheilerial drug ^993C (Wellcome Research Laboratories, Beckenham) and subsequently resisted homologous stabilate challenge on 11.3.82. Both calves were splenectomized on day 69 after the initial infection (21.4.82), as described in Section 2.3, to precipitate a recrudescence in the piroplasm parasitaemia.

(b) Recipient calves - Calves 163 and 164 were splenectomized by the same procedure as the donors, 34 days before the inoculation of parasitized blood.

6.2.2 Transmission by blood inoculation: One hundred ml of blood with a parasitaemia of 12-14% was aseptically removed from calf 155 by jugular venepuncture on 19.5.82 (28 days post splenectomy) and defibrinated. After thorough mixing, 20 ml of blood was aspirated into one syringe and held at room temperature until inoculated into calf 164. A 30 ml aliquot of blood was aspirated into a second syringe and passed in 5 ml aliquots through six 25 mm Swinnex filters each containing an AP prefilter and 7μ* filter (Millipore). The pooled filtrate was then passed in 5 ml aliquots through six additional 7μ* filters.

Calf 163 received 12 ml of the filtered blood intravenously and 12 ml subcutaneously dorsal to the right prescapular gland (RPG). Calf 164 received 10 ml of unfiltered blood intravenously and 10 ml subcutaneously dorsal to the RPG. The time from blood collection to inoculation was approximately one hour.

6.2.3 Evaluation of inocula: Erythrocytes and leucocytes were counted on an electronic particle counter. Smears were prepared from fresh whole blood and aliquots of the two inocula, stained with Giemsa and examined for parasites (Section 2.12.1).

*nylon Duralon NS filters.

Stationary erythrocyte cultures were established with 3% (v/v) suspensions of concentrated erythrocytes, obtained as described in Section 2.8.2 from the defibrinated blood, and from a 1:20 dilution of the final filtered blood. The complete medium consisted of 60% Medium 199 and 40% foetal bovine serum. Five ml of each suspension were deposited into two vertical culture flasks, gassed with 5% CO_2 and air, and maintained as described in Section 2.8.3. Cultures were evaluated by counting the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites within 100 PRBC on Giemsa stained cytocentrifuge smears which were prepared from each flask on days 0, 2 and 6 in vitro.

Lymphoid cell cultures were established as described in Section 2.11 with 10 ml aliquots of heparinized and defibrinated blood taken from calf 155 on the day of the transmission to the recipient calves.

6.2.4 Monitoring infections: Routine monitoring procedures were conducted as described in Section 2.6.2. Weekly biopsies were taken alternately from the left (LPG) and right (RPG) prescapular lymph nodes of carrier calves 154 and 155. The RPGs of the two recipients, 163 and 164, were biopsied weekly to day 35 post-infection and subsequently both prescapular lymph nodes were sampled weekly. Fresh blood smears were prepared every one to two days during the designated evaluation period. All smears were stained with Giemsa and examined for parasites (Section 2.11.1).

Lymphoid cell cultures were established with samples from the spleens of 154 and 155 after surgical removal, and from the livers of calf 163 biopsied on days 35 and 47 post-splenectomy and calf 164 biopsied on day 35 post-splenectomy, by the method in Section 2.10.

Peripheral blood lymphocytes were isolated *in vitro* as described in Section 2.11 from all four calves each week and from the donor calf 155 on the day of blood transmission. Giemsa stained cytocentrifuge smears were prepared with aliquots of the lymphoid cell suspensions on the day of culture establishment.

Selected serum samples from the four calves were evaluated for the presence of antibodies to macroschizont and piroplasm antigens by the indirect fluorescent antibody test (Section 2.6.2).

6.2.5 Poisson distribution analysis of parasite counts: Counts were made of the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites within 500 PRBC in Giemsa stained fresh blood smears prepared on selected days from both the carrier and the recipient calves.

The total number of parasites per 500 PRBC and the total number of erythrocytes counted to detect 500 PRBC were used to calculate the average number of parasites per erythrocyte (μ). The formula used to calculate the frequency of specific numbers of intraerythrocytic parasites expected to occur, based on Poisson distribution is:

$$P(r) = \frac{\mu^r}{r!} e^{-\mu}$$

P = probability

r = observed frequency of the event

μ = average number of parasites per erythrocyte

e = 2.71828...

The observed frequency (O) of 0, 1, 2, 3, 4 ..... 12 intraerythrocytic parasites was compared to the expected frequency (E) with the Chi-square (χ^2) test (Snedecor and Cochran, 1980). The χ^2 values ($\chi^2 = \frac{(O-E)^2}{E}$) for the different numbers of intraerythrocytic

parasites observed were considered in evaluations, in addition to the total χ^2 values for each day, so as to identify where the greatest variation in the incidence of parasites occurred. The observed and expected values were converted to standard values per 1000 erythrocytes for display in the figures and appendices.

6.3 Results

6.3.1 Reactions in carrier calves 154 and 155: The thermal, haematological and parasitic reactions observed in calves 154 and 155 post splenectomy are illustrated in Figures 6.1 and 6.2. The results of the indirect fluorescent antibody test using sera from these calves are given in Table 6.1.

In both calves the initially low piroplasm parasitaemias increased after splenectomy to a peak on day 25, followed by a gradual decline. There was a corresponding reduction in the packed cell volumes and erythrocyte counts, as the parasitaemia increased, particularly in calf 155 after day 25. The number of leucocytes in the peripheral blood varied within a normal range in both calves after splenectomy.

Other than a brief rise immediately after surgery, the only temperature elevation greater than 39.5°C occurred on days 28-30, after the peak parasitaemia and coinciding with a reduction in the PCV in both calves.

T. annulata-infected lymphoid cells were not detectable in blood or lymph node biopsy smears prepared from either calf after splenectomy. Schizont infected lymphoid cells were, however, isolated intermittently from peripheral blood samples of both calves throughout the experiment (Figures 6.1 and 6.2).

Figure 6.1 Clinical and parasitic reactions after splenectomy of Theileria annulata (Ankara) carrier calf 154.

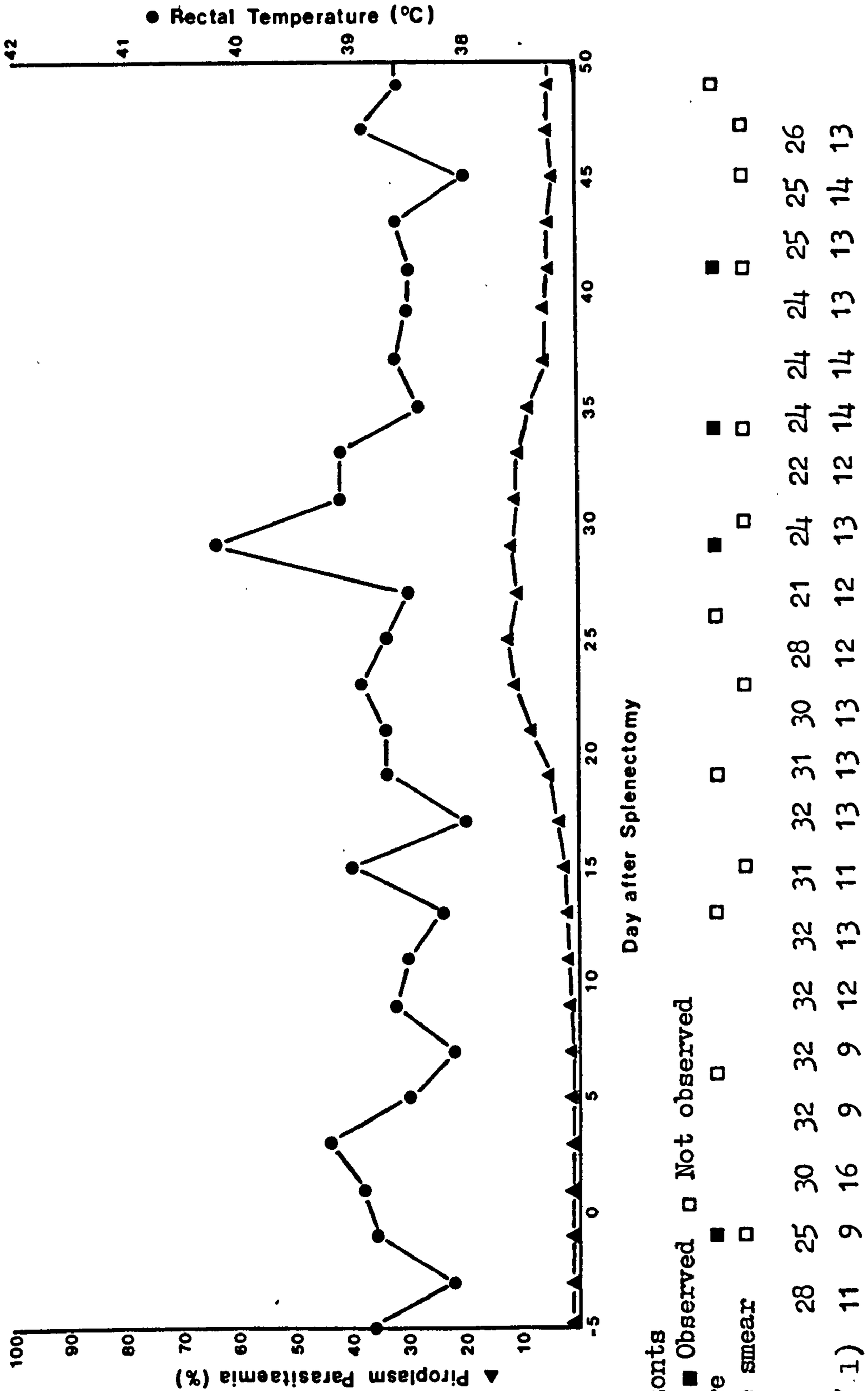

● Rectal Temperature (°C)
42
41
40
39
38
▲ Piroplasm Parasitaemia (%)
100
90
80
70
60
50
40
30
20
10
50
45
40
35
30
25
20
15
10
5
0
-5
Day after Splenectomy
□ Not observed
Macroschizonts
■ Observed □
PBL culture
■
Lymph node smear
□
PCV (%) 28 25 24 25 25 26
WBC (x10⁹/l) 11 9 13 13 14 13
30 28 21 24 24 24
13 12 12 13 12 14
30 32 32 32 31 32
16 9 9 12 13 13
30 32 32 32 31 31
9 9 12 13 11 13

Figure 6.2 Clinical and parasitic reactions after splenectomy of Theileria annulata (Ankara) carrier calf 155.

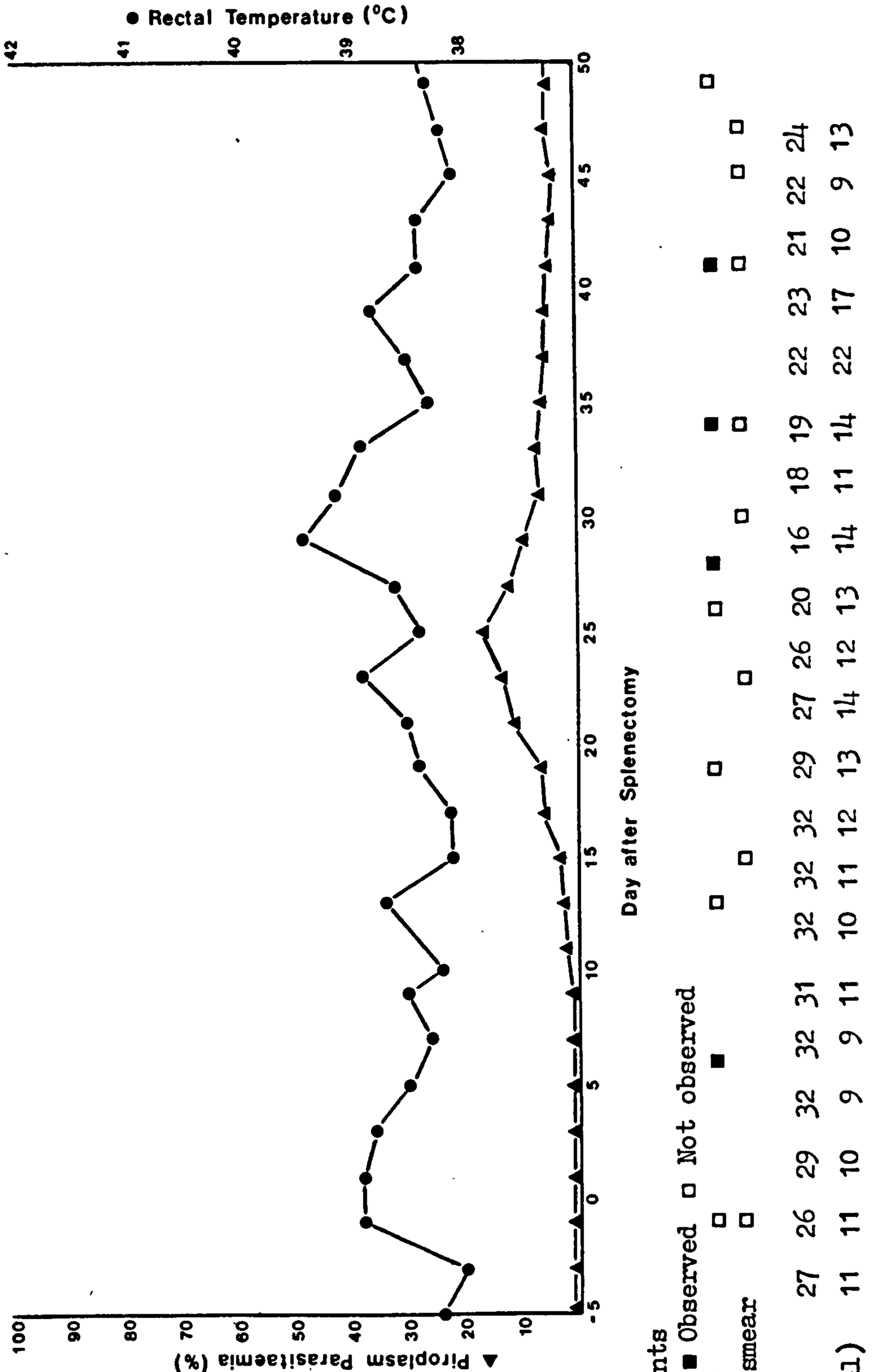

● Rectal Temperature (°C)
42
41
40
39
38
50
45
40
35
30
25
20
15
10
5
0
-5
Day after Splenectomy
▲ Piroplasm Parasitaemia (%)
100
90
80
70
60
50
40
30
20
10
Macroschizonts ■ Observed □ Not observed
PBL culture
Lymph node smear
PCV (%) 27 26 29 32 32 31 32 32 32 32 29 24
WBC (x10⁹/l) 11 11 10 9 9 11 10 11 12 13 13 13
□ 23 21 22 24
■ □ 17 10 9 13
□ 27 26 20 16 18 19 22 22
■ □ 14 12 13 14 11 14 22 22

Table 6.1 Antibody titres of calves 154 and 155 to Theileria annulata (Ankara) macroschizont and piroplasm antigens determined by the indirect fluorescent antibody test

Date of serum sample	Day after infection	Day after challenge	Day after splenectomy	Calf 154		Calf 155	
				Macroschizont antigen	Piroplasm antigen	Macroschizont antigen	Piroplasm antigen
10/2	0	0	0	1:40	1:40	1:40	<1:40
1/4	49	21	0	1:2560	1:2560	1:2560	1:640
16/4	64	36	0	1:2560	1:2560	1:2560	1:640
6/5	84	56	15	1:2560	1:2560	1:2560	1:640
19/5	97	69	28	1:640	1:640	1:2560	1:640
27/5	105	77	36	1:640	1:640	1:2560	1:160
4/6	113	84	44	1:640	1:640	1:2560	1:640
12/6	121	92	52	1:640	1:640	1:2560	1:640

Both calves 154 and 155 had appreciable antibody titres to macroschizont and piroplasm antigens which were not affected by splenectomy (Table 6.1). The reduction in the antibody levels between days 28 and 36 corresponded to the period of declining piroplasm parasitaemia.

6.3.2 Evaluation of inocula: Erythrocyte, leucocyte and parasite counts from samples of the two inocula given to calves 163 and 164 are displayed in Table 6.2. Parasite counts of the samples from stationary erythrocyte cultures established with the inocula are displayed in Table 6.3.

The number of erythrocytes per ml were similar in the two inocula (Table 6.2). The number of leucocytes in the filtered blood was too low to be accurately counted by the automated particle counter. Therefore, five Giemsa stained smears were prepared with 10 µl aliquots of the filtered blood and carefully examined for the presence of leucocytes. A total of ten small lymphocytes were seen on the five smears (total 50 µl). The number of leucocytes observed on similar smears prepared with unfiltered blood were too numerous to warrant counting .

The intraerythrocytic parasites were morphologically similar in smears prepared with the two inocula prior to transmission and after two to six days in vitro. The number of quadruplet forms and parasitized erythrocytes in the cultures established with suspensions of the two inocula were comparable (Table 6.3).

The lymphoid cell cultures established with the unfiltered defibrinated blood had to be discarded due to fungal contamination.

Table 6.2 Evaluation of Theileria annulata (Ankara) infected blood inocula used to infect calves 163 and 164

Inoculum sample	Number of parasites in erythrocyte* 1	2	3	4	5	PRBC* per 1000 erythrocytes	Erythrocytes** x 10^9/ml	Leucocytes** x 10^6/ml
Filtered blood inoculum to calf 163	450	35	3	9	3	140 ± 7	4.43	0.07
Unfiltered blood inoculum to calf 164	456	33	1	10	0	120 ± 9	4.45	5.50

*Figures are pooled counts of parasites in 500 parasitized erythrocytes (PRBC) and the mean ± standard deviation of PRBC per 1000 erythrocytes in three samples.

**Figures displayed for erythrocyte and leucocyte counts are after correction (electronic particle counter isoton background = 0.20).

Table 6.3 Evaluation of Theileria annulata (Ankara) infected blood inocula maintained in stationary erythrocyte cultures

Inoculum sample	Day in vitro	Number of parasites in erythrocyte								PRBC per 1000 erythrocytes
		1	2	3	4	5	6	7	8	
Filtered blood inoculum to calf 163	0	91	6	0.5	2	0.5	0	0	0	140
	2	73	5	1	19	1.5	0	0	0.5	125
	6	70	7	0	21	2	0	0	0	100
Unfiltered blood inoculum to calf 164	0	93	5	0	2	0	0	0	0	120
	2	75	6	0	17	1	1	0	0	135
	6	66	7	3	22	1	1	0	0	114

Figures are mean counts of parasites in 100 parasitized erythrocytes (PRBC) and number of PRBC per 1000 erythrocytes in samples from the inocula (Day 0) and two cultures on day 2 and 6 in vitro.

Isolations made *in vitro* from the heparinised blood sample taken from calf 155 at the same time showed detectable macroschizont-infected lymphoid cells within five days after culture establishment. By day 19 *in vitro* over 50% of the lymphoid cells in these cultures contained macroschizonts.

6.3.3 Reactions in recipient calves 163 and 164: The thermal, haematological and parasitic reactions in calves 163 and 164 after inoculation with blood from calf 155 are displayed in Figures 6.3 and 6.4. The results of serological evaluations using the indirect fluorescent antibody test are summarised in Table 6.4.

Piroplasms were detected in the blood of both calves within four hours of inoculation. Calf 164 had at least ten times more parasitized erythrocytes than calf 163 during the first two weeks post-infection when the parasitaemias of both calves were below 0.1%. The general pattern of the parasitic response was similar in the two calves but the onset of a parasitaemic rise (above 0.1%), the first parasitaemic peak (above 10%) and the first detectable macroschizonts occurred 8-14 days earlier in the course of the infection in calf 164 which received unfiltered blood.

The erythrocyte concentration and packed cell volume decreased gradually in both calves as the parasitaemia increased (Figures 6.3 and 6.4).

Macroschizont-infected lymphoid cells were not detected until after the first parasitaemic peak in both calves. Macroschizont-infected cells were observed in the RPG biopsy smear and isolated *in vitro* from the peripheral blood of 164 on day 33 post infection.

Figure 6.3 Clinical and parasitic reactions of calf 163 after inoculation of filtered Theileria annulata (Ankara) infected blood.

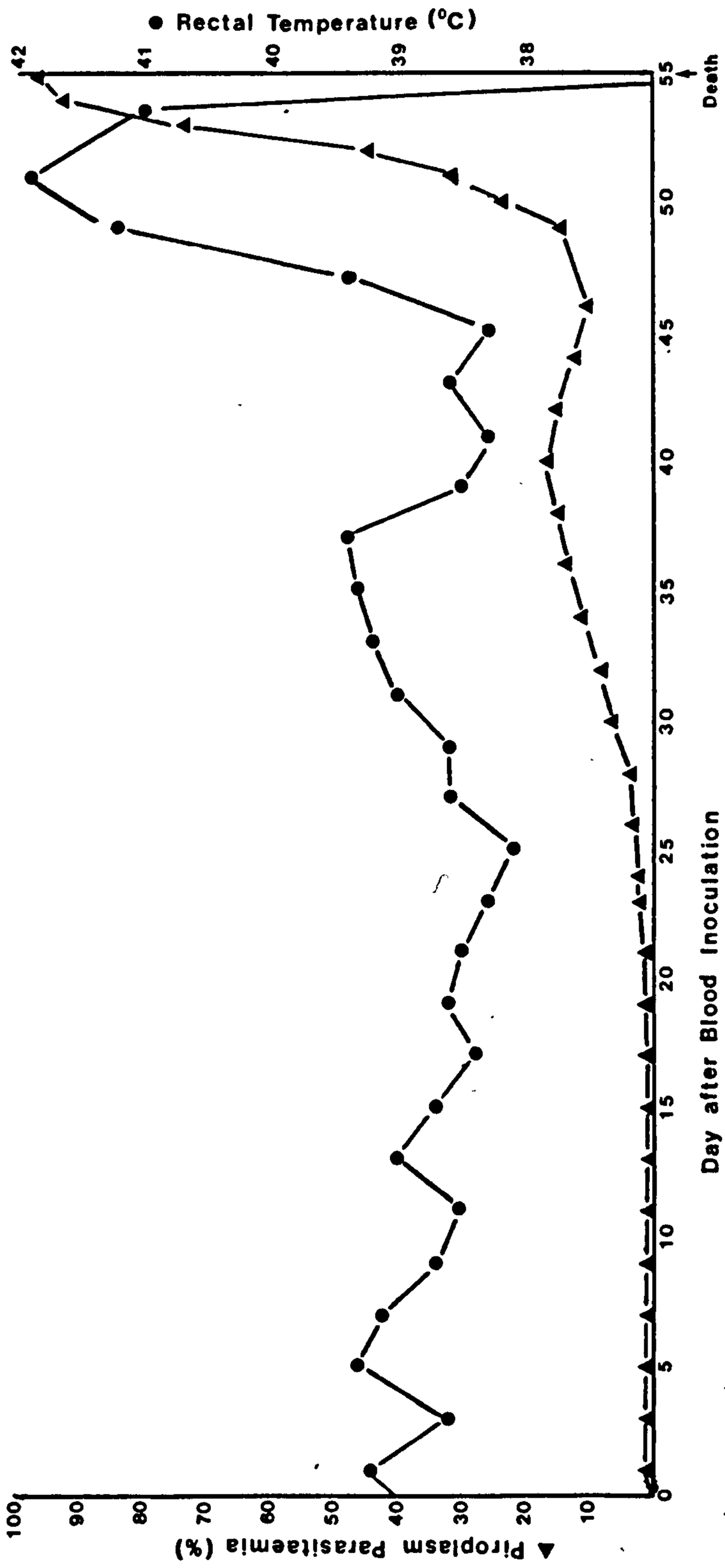

● Rectal Temperature (°C)
42
41
40
39
38
55
Death
50
45
40
35
30
25
20
15
10
5
0
Day after Blood Inoculation
▲ Piroplasm Parasitaemia (%)
100
90
80
70
60
50
40
30
20
10
□ Not observed
■ Observed
Macroschizonts
PBL culture
Lymph node smear
Liver smear
PCV (%)
WBC (x10⁹/ 1)
38 35 26 29 27 26 8 11 9 14 18 16 32 35 32 34 37
10 11 8 13 10 13 13 20 14 12 23 7 5 19 6 3

Figure 6.4 Clinical and parasitic reactions of calf 164 after inoculation of unfiltered Theileria annulata (Ankara) infected blood.

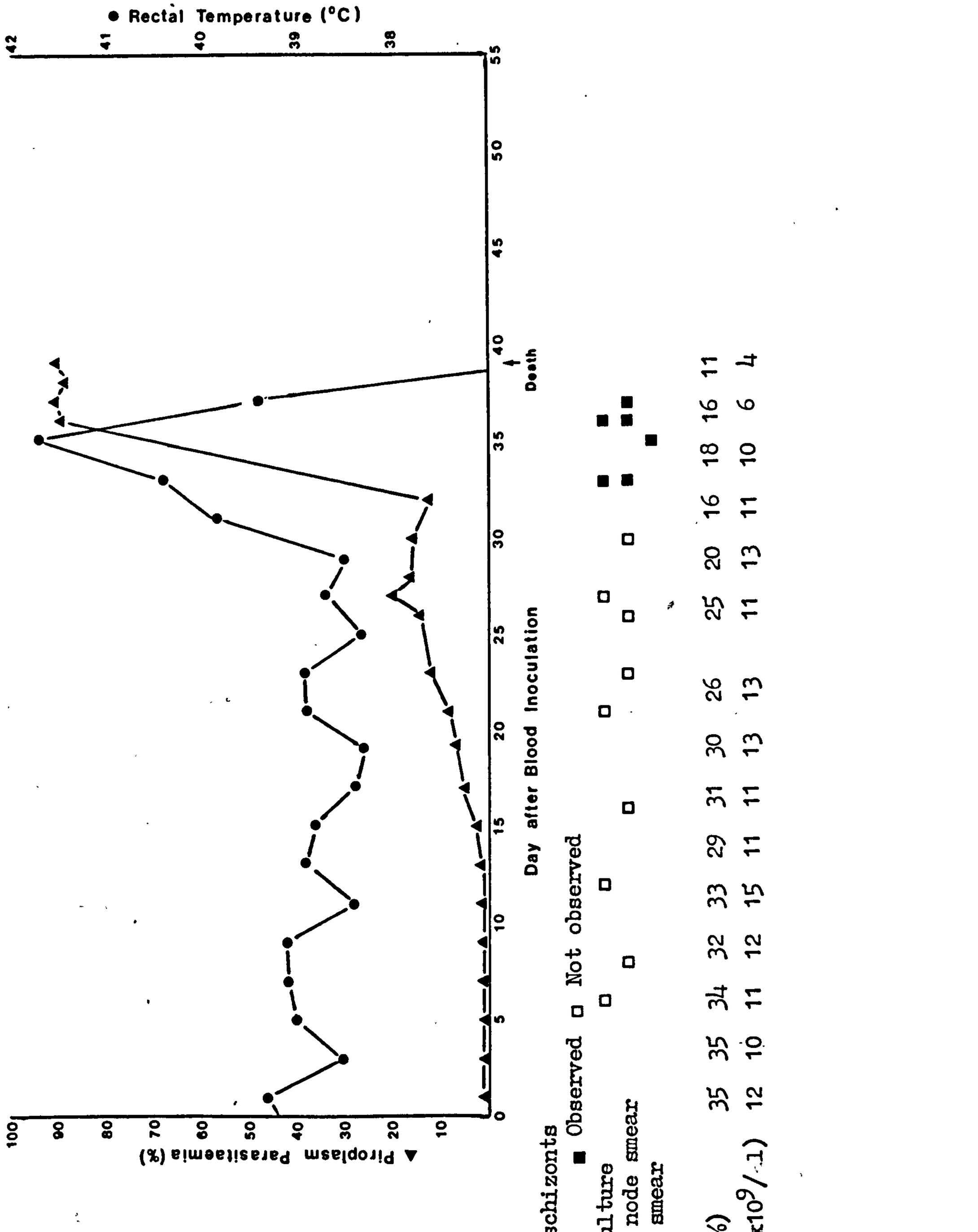

● Rectal Temperature (°C)
42
41
40
39
38
55
50
45
40
35
30
25
20
15
10
5
0
Death
Day after Blood Inoculation
▲ Piroplasm Parasitaemia (%)
100
90
80
70
60
50
40
30
20
10
Macroschizonts
■ Observed □ Not observed
PBL culture
Lymph node smear
Liver smear
PCV (%)
WBC (x10⁹/l)
26 25 20 16 18 16 11
13 11 13 11 10 6 4
34 32 33 29 31 30
11 12 15 11 11 13
35 35
12 10

Table 6.4 Antibody titres of calves 163 and 164 to Theileria annulata (Ankara) macroschizont and piroplasm antigens determined by the indirect fluorescent antibody test

Date of serum sample	Day after blood inoculation	Calf 163 inoculated with filtered blood		Calf 164 inoculated with unfiltered blood	
		Macroschizont antigen	Piroplasm antigen	Macroschizont antigen	Piroplasm antigen
19.5	0	<1:40	<1:40	<1:40	<1:40
27.5	8	<1:40	1:160	1:160	1:160
4.6	16	1:40	1:640	1:640	1:2560
11.6	23	1:160	1:10240	1:640	1:2560
18.6	30	1:640	1:10240	1:2560	1:2560
23.6	35	1:640	1:10240	1:2560	1:2560
25.6	37				
30.6	42	1:640	1:10240	1:10240	1:2560
5.7	47	1:640	1:10240	1:10240	1:2560
10.7	52	1:640	1:2560		
12.7	54	1:2560	1:2560		

In calf 163 T. annulata-infected lymphoid cells were isolated from the peripheral blood on day 41 post infection and later identified in lymph node and liver biopsy samples on day 47. The appearance of macroschizonts in both calves immediately preceded a second parasitaemic peak which was coincident with pyrexia, leucopenia and death.

6.3.4 Comparison between the observed and expected distribution of intraerythrocytic parasites:

(a) Carrier calves 154 and 155 - Counts made of intraerythrocytic parasites in smears prepared with fresh blood from T. annulata (Ankara) carrier calves 154 and 155 on selected days post-splenectomy are shown in Appendices 10 and 11.

The incidence of erythrocytes with specific numbers of parasites observed in the blood are compared, in Appendices 12 and 13, to the frequency of multi-parasitized erythrocytes that would be expected to occur in a Poisson distribution. The greatest difference between the observed and expected values occurred when four or five parasites were seen inside erythrocytes.

The number of erythrocytes seen to contain four parasites was less than the incidence of erythrocytes with two parasites, as shown in Figures 6.5 and 6.6 (note the log difference in the y-axis scale on these figures). The observed incidence of erythrocytes with four parasites was, however, highly significant when compared to the expected values. The only exception was seen on day 24 post splenectomy as the parasitaemia in calf 155 reached 14%. Although one quadruplet form was seen in 500 parasitized erythrocytes on that day

the difference between the observed and expected value was not significant based on the Chi-square test.

(b) Recipient calves 163 and 164 - Counts made of intraerythrocytic parasites in fresh blood smears from calves 163 and 164 prepared on selected days post-infection are displayed, respectively, in Appendices 14 and 15. The observed distributions of parasitized erythrocytes are compared to the Poisson distribution in Appendices 16 and 17. Figures 6.7 and 6.8 illustrate the relationship between the observed and expected incidence of erythrocytes containing two or four parasites.

The highly significant χ^2 values in blood sampled when the parasitaemia was below 20% were due primarily to the difference between the expected number of erythrocytes with four or five parasites and the higher observed incidence of these multi-parasitized cells (Appendices 14-17). At the higher parasitaemias, in calf 163 after day 46 and in calf 164 after day 32, the incidence of erythrocytes without parasites, as well as with numbers of parasites other than four or five, contributed to the high total χ^2 values. The parasitaemic rise above 20% was coincident with the appearance of macroschizont-infected lymphoid cells in both calves.

The observed incidence of erythrocytes with two parasites was never significantly greater than the expected incidence, based on Poisson distribution, in the blood samples from both calves. The number of erythrocytes with four parasites seen in the blood prior to the second parasitaemic rise was highly significant when compared to the expected incidence (Figures 6.7 and 6.8).

Figure 6.5 Incidence of erythrocytes with 2 or 4 parasites observed in the blood of calf 154 compared to expected Poisson distribution values.

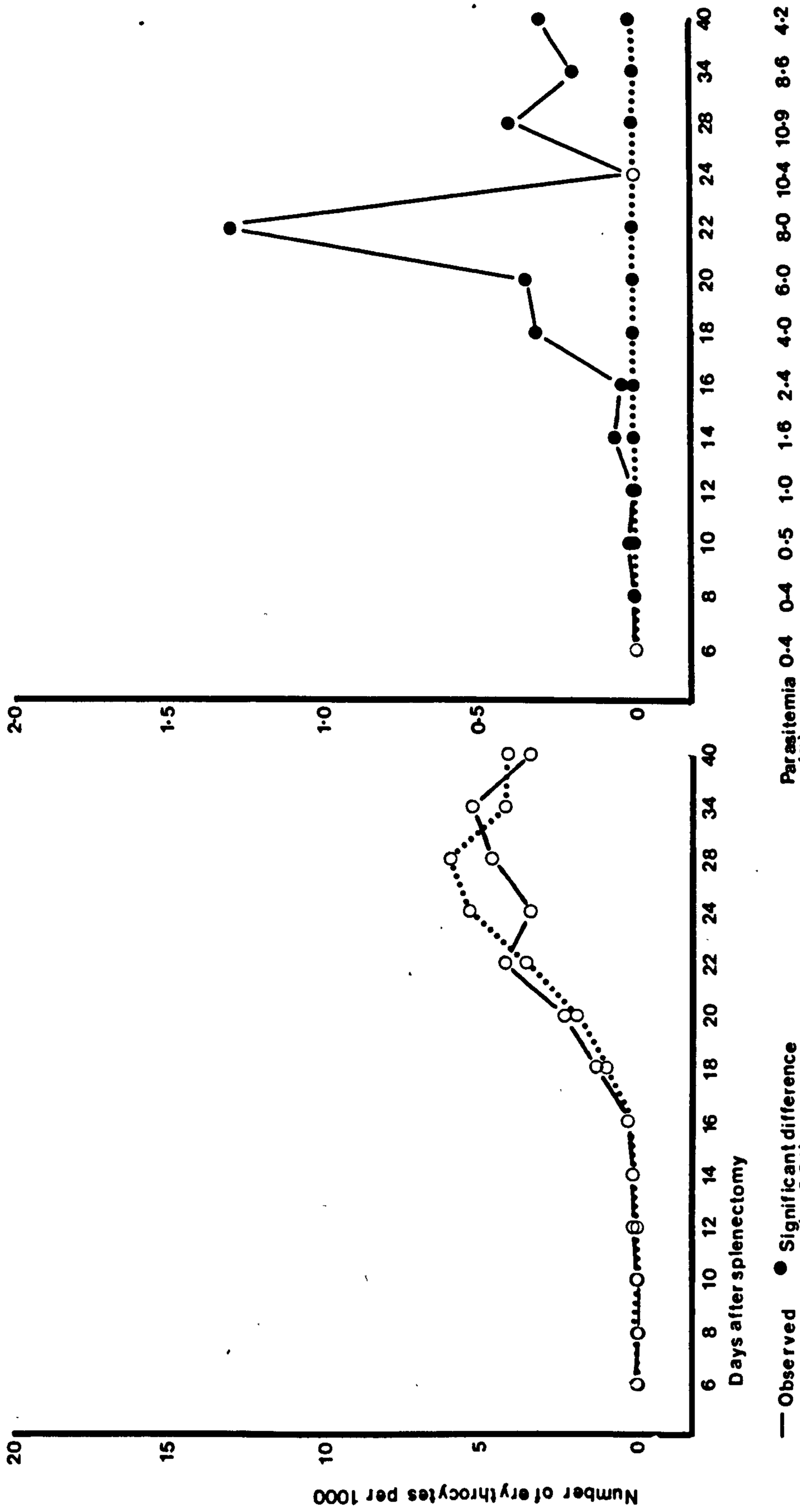

4 PARASITES
2 PARASITES
Number of erythrocytes per 1000
Parasitemia 0·4 0·4 0·5 1·0 1·6 2·4 4·0 (%)
6·0 8·0 10·4 10·9 8·6 4·2
Days after splenectomy
Observed
Expected
Significant difference (p<0·01)
Not significant

Figure 6.6 Incidence of erythrocytes with 2 or 4 parasites observed in the blood of calf 155 compared to expected Poisson distribution values.

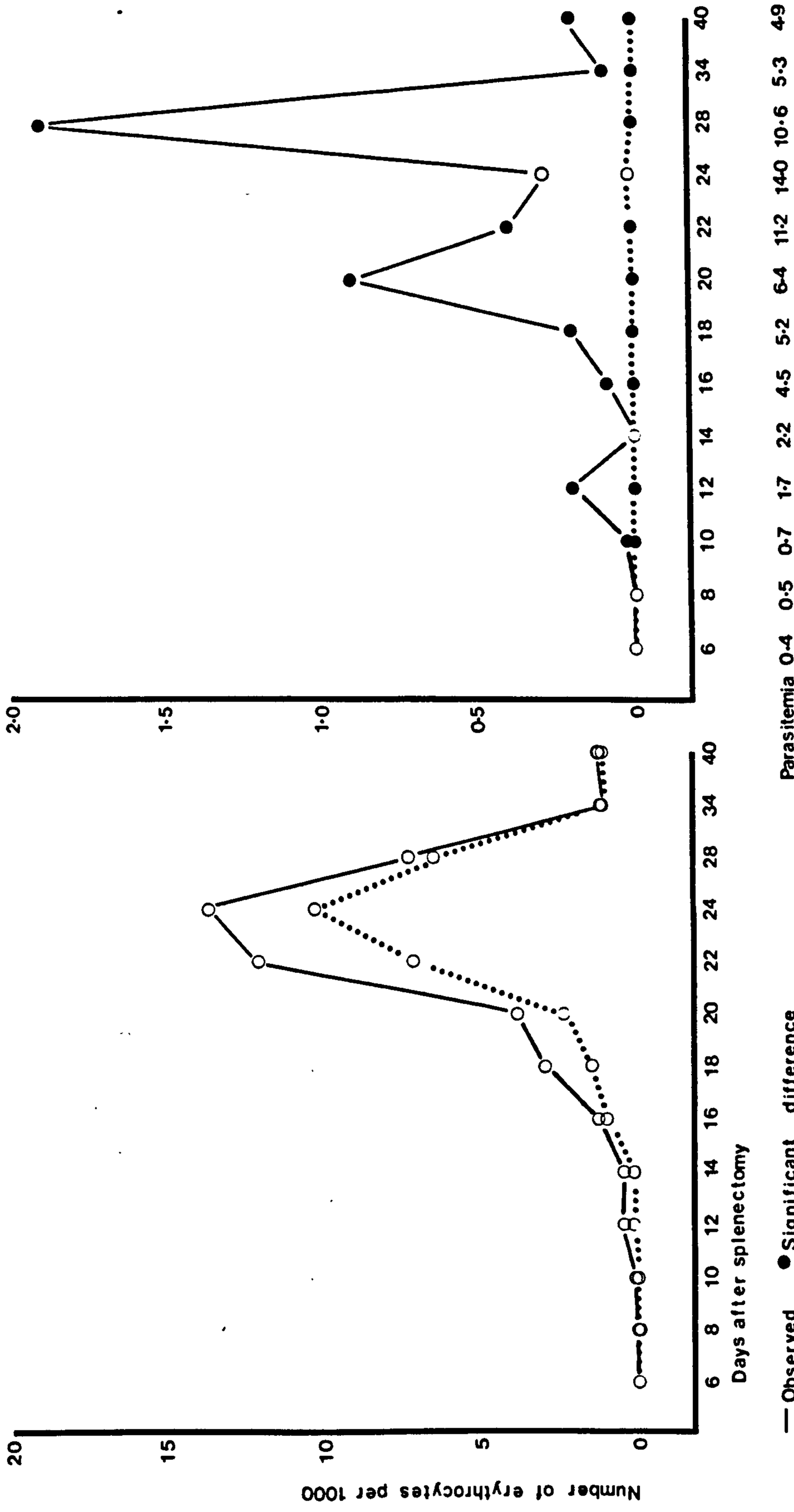

4 PARASITES
2 PARASITES
Number of erythrocytes per 1000
Parasitemia (%)
0·4 0·5 0·7 1·7 2·2 4·5 5·2 6·4 11·2 14·0 10·6 5·3 4·9
Days after splenectomy
Observed
Expected
Significant difference (p<0·01)
Not significant

Figure 6.7 Incidence of erythrocytes with 2 or 4 parasites observed in the blood of calf 163 compared to expected Poisson distribution values.

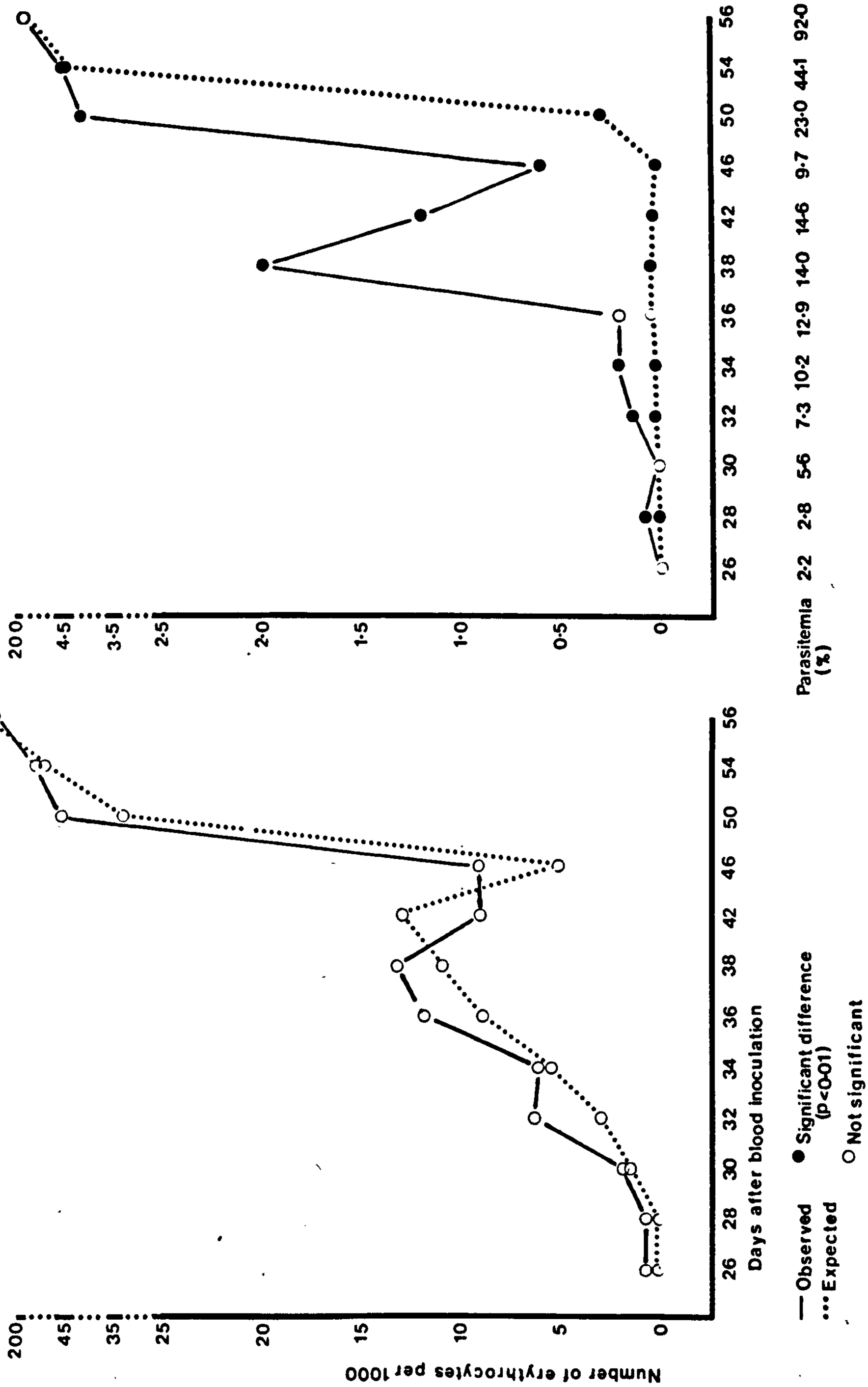
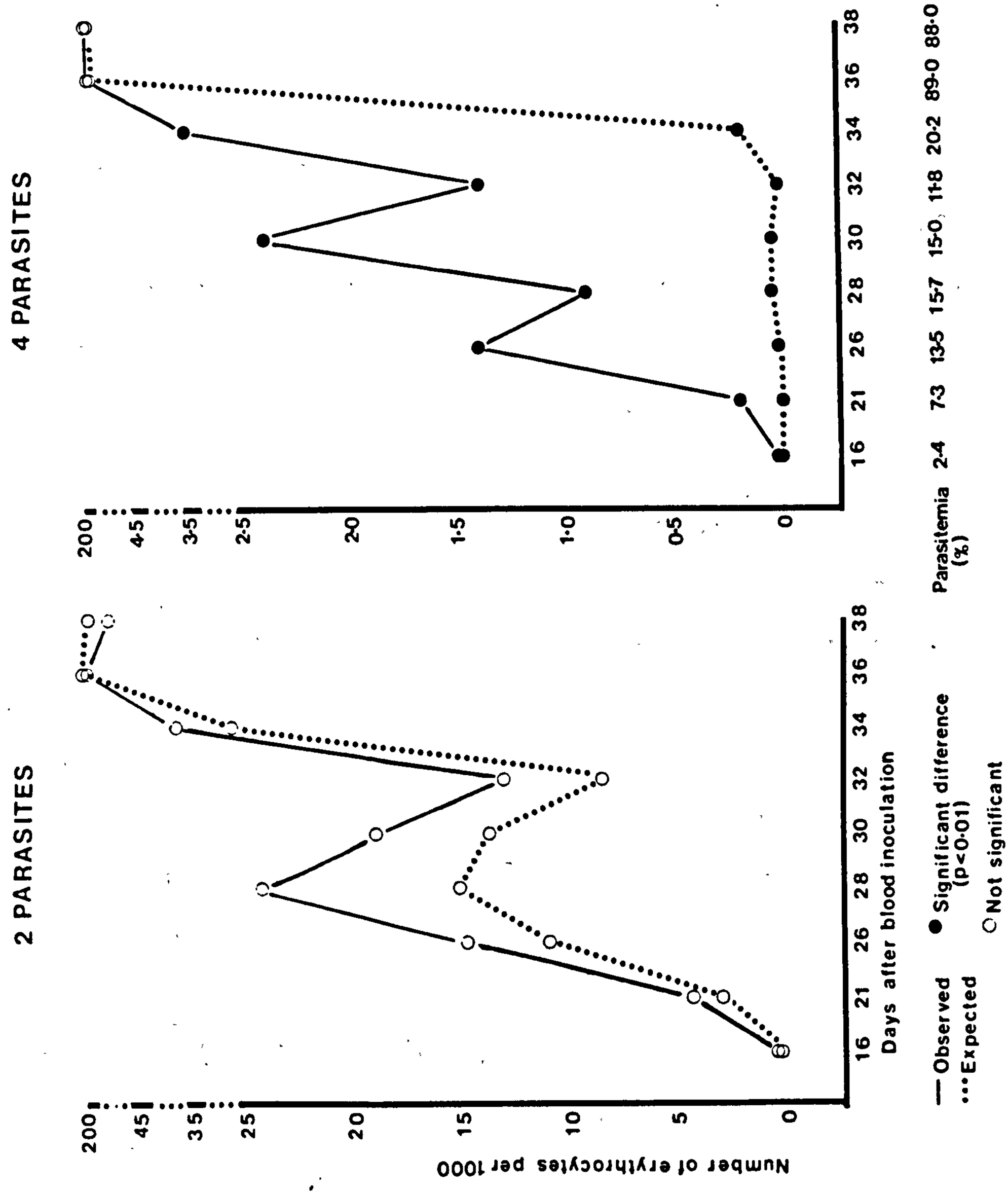

4 PARASITES
2 PARASITES
200
4·5
3·5
2·5
2·0
1·5
1·0
0·5
0
Number of erythrocytes per 1000
26 28 30 32 34 36 38 42 46 50 54 56
Days after blood inoculation
Parasitemia (%) 2·2 2·8 5·6 7·3 10·2 12·9 14·0 14·6 9·7 23·0 44·1 92·0
— Observed
••• Expected
● Significant difference (p<0·01)
○ Not significant

Figure 6.8 Incidence of erythrocytes with 2 or 4 parasites observed in the blood of calf 164 compared to expected Poisson distribution values.


4 PARASITES
2 PARASITES
Number of erythrocytes per 1000
200
4·5
3·5
2·5
2·0
1·5
1·0
0·5
0
45
35
25
20
15
10
5
0
16 21 26 28 30 32 34 36 38
Days after blood inoculation
Parasitemia (%) 2·4 7·3 13·5 15·7 15·0 11·8 20·2 89·0 88·0
— Observed
••• Expected
● Significant difference (p<0·01)
○ Not significant

6.4 Discussion

The experimental protocol was designed with the intention of separating the piroplasm stages of T. annulata (Ankara) from the intralymphocytic stages, so as to study the mode of intraerythrocytic multiplication in vivo. In the course of this experiment valuable information was obtained regarding the nature of the carrier state in T. annulata infections and the effect of splenectomy on carrier calves, as well as on the behaviour of the intraerythrocytic parasites. This discussion will deal first with the observations relevant to the primary aim of the experiment. The reactions in the carrier calves and their significance will then be discussed.

Despite the precautions intended to assure that the recipient calves received only the intraerythrocytic stages of T. annulata, the possibility existed that macroschizont-infected lymphoid cells were present in the inocula. Macroschizonts had not been found in the peripheral blood lymphocyte(PBL) isolations from the potential donors, calves 154 and 155, prior to the day that the recipients were infected. However, on the day of the transmission, schizonts were identified in the PBL isolate from the selected donor, calf 155. The unfiltered and perhaps, the filtered blood inocula may have contained infected lymphoid cells. Calf 163 received an additional 4 ml of blood, therefore more parasitized erythrocytes, but at least 10^4 fewer leucocytes than calf 164 (Table 6.2). The piroplasms in the two inocula were similar in number and appeared to be equally capable of multiplying in vitro, based on their behaviour in stationary erythrocyte cultures (Table 6.3).

The reactions in the two recipient calves were strikingly similar with two peaks in the piroplasm parasitaemia, the latter accompanied by the appearance of macroschizonts, pyrexia, leucopenia, severe anaemia and death (Figures 6.3 and 6.4). The piroplasm parasitaemia in calf 164 did, however, begin to increase and reach its two peak levels 7-14 days earlier than in calf 163. Macroschizonts were isolated from the peripheral blood eight days earlier in calf 164 than in calf 163. The level of antibodies to macroschizont antigens detected in the blood of calf 164 also began to increase earlier and remained at a higher level throughout the rest of the sample period than the antibody levels detected in the serum of calf 163.

The difference in the timing of the reactions observed in the two recipients could be attributed to the fact that calf 164 may have received more macroschizont-infected lymphoid cells than calf 163. One conclusion from the observations made in this in vivo study might be that the piroplasm parasitaemia in the carrier and recipient calves was maintained by the continual invasion of erythrocytes by merozoites formed from intralymphocytic schizonts.

The fact that macroschizont-infected lymphoid cells were not detected either in the peripheral blood isolations in vitro or in lymph node biopsy smears until after the first peak in the piroplasm parasitaemia suggests a second theory. The merozoites formed both from intralymphocytic and intraerythrocytic schizogony could be capable of invading erythrocytes, thus jointly contributing to the piroplasm parasitaemia. In support of this theory is the observed increase in the incidence of erythrocytes with quadruplet forms which coincided with the initial increase in the piroplasm parasitaemia in

the carrier calves (Appendices 10 and 11) and recipient calves (Appendices 14 and 15). Electron microscopic studies in Chapter 5 have shown that quadruplet forms represent merozoites resulting from a process of intraerythrocytic schizogony.

Comparisons were made between the observed incidence of erythrocytes with specific numbers of parasites and Poisson distribution, so as to determine whether intraerythrocytic multiplication was occurring and if so, the number of merozoites formed in the process. Poisson distribution is used to predict the probability of rare events, where the binomial distribution would be markedly skewed. The best application of Poisson distribution is when the sample size tends towards infinity and the probability of the specific event occurring tends towards zero.

In evaluations of low parasitaemia blood samples, Poisson distribution could be applied to determine whether the distribution of erythrocytes with specified numbers of parasites corresponded to the distribution which might occur from the random invasion by exoerythrocytic merozoites. The probability of erythrocytes with four or five parasites occurring in low parasitaemia blood solely as a result of invasion from exoerythrocytic merozoites is extremely low. The presence of these cells with four or five parasites, in the blood of both the carriers and recipients was, therefore, highly significant. The erythrocytes seen to contain five parasites invariably included a single piroplasm and a quadruplet form.

The number of erythrocytes with two parasites was greater than the number with four, but not greater than the number expected to occur by chance in a Poisson distribution (Figures 6.5-6.8). The

indication was that division into four rather than binary fission is the primary mode of multiplication *in vivo* for the intraerythrocytic stages of *T. annulata*.

The second parasitaemic peak in the recipients appeared to be primarily due to the invasion of exoerythrocytic merozoites from microschizonts. Macroschizonts and microschizonts were observed in the lymphoid cells of both calves when the piroplasm parasitaemias began to rise above 20%. The increase in the average number of parasites per erythrocyte at this high parasitaemia meant that the incidence of multi-parasitized erythrocytes could no longer be expected to fit Poisson distribution.

Interpretation of the results in this experiment must take into consideration a third intriguing possibility, namely that merozoites produced by intraerythrocytic multiplication may be capable of invading erythrocytes, lymphocytes and/or other, as yet unidentified, cells. The first peak in the piroplasm parasitaemia in the recipient calves could have resulted primarily from intraerythrocytic multiplication. Merozoites formed by intraerythrocytic schizogony might be capable of invading other cells, including lymphocytes. Schizonts formed in these cells could have produced merozoites that invaded erythrocytes, resulting in the second parasitaemic peak.

The theory has been previously proposed that piroplasms of *T. annulata* (Pipano, 1972) and *T. mutans* (Oteng, 1972) are capable of reinvading lymphoid cells. Although this supposition lacks the experimental evidence for confirmation, the possibility that such a phenomenon occurs cannot be discounted. Development of the parasite within different cell types could assist the parasite in escaping

the host's protective immune response and more effectively assure the persistence of infection.

Overall, the second theory, that merozoites formed from both intralymphocytic and intraerythrocytic schizonts invade erythrocytes to form piroplasms, seems to be most tenable. The three hypotheses cannot, however, be satisfactorily tested nor the kinetics of replication be accurately evaluated for the intraerythrocytic stages of T. annulata in vivo by the protocol employed, unless all other potentially parasitized cells and free exoerythrocytic merozoites can be completely removed from the blood inocula.

Separating the intracellular stages in the mammalian phase of the Theileria life cycle proved to be difficult, but observations made during the attempt, described in this chapter, suggested interesting areas for further study. Of particular interest were the parasitic reactions in carrier calves 154 and 155 after splenectomy. Removal of the spleen has been reported to result in a recrudescence of the piroplasm parasitaemia in T. annulata-infected cattle but the effect on the macroschizont stage has not been well documented (Sergent et al., 1945; Hooshmand-Rad, 1976; Srivastava and Sharma, 1976b).

After splenectomy the piroplasm parasitaemia rose gradually in calves 154 and 155 to a peak level by day 25. The prevalence of macroschizont-infected lymphoid cells in the blood appeared to increase between days 28 and 44 post-splenectomy. The basis for this statement is the observation that infected lymphoid cells appeared within one week in vitro in cultures established with peripheral blood taken between days 28 and 44 post-splenectomy from the two

calves. Some of the lymphocyte isolations made from two weeks to one day prior to splenectomy contained macroschizont-infected cells, but a two to three week cultivation period was required before the number of infected cells reached a detectable level.

Judging by the response to splenectomy, some of the immunological factors which suppressed the piroplasm parasitaemia in the carrier calves may also have an effect on the intralymphocytic macroschizonts.

Acquired immunity is associated with a cell-mediated response in infections with T. parva (Pearson et al., 1979; Emery and Morrison, 1980) and T. annulata (Rehbein, Ahmed, Schein, Hörchner and Zweygarth, 1981; Preston et al., 1983; Musiime, 1983). At least two types of effector cells are apparently involved in the destruction of schizont-infected lymphoblastoid cells, as both genetically restricted and non-specific cytotoxicity is generated by lymphocytes from cattle recovering from theileriosis (Pearson et al., 1979; Emery et al., 1981a; Emery, Eugui, Nelson and Tenywa, 1981; Preston et al., 1983; Emery and Kar, 1983).

Preston et al. (1983) found that after the initial stabilate infection of calves 154 and 155, the first peak of cytolysis was specifically directed towards T. annulata (Ankara) derived or induced antigens associated with the major histocompatibility (BoLA) antigens on allogeneic cell lines, whereas subsequent cytotoxicity did not appear to be genetically restricted. The less specific, cell-mediated response which occurred later in the course of the infection is particularly interesting because this may involve immune factor(s) which inhibit both the intralymphocytic and intraerythrocytic stages of

Theileria. These factors could play an important role in the control of chronic theilerial infections.

The cytotoxicity directed against Theileria-transformed lymphoblastoid cells is similar to the response generated against neoplastic cells, which has been attributed to activated macrophages and natural killer (NK) cells (Hanna, 1980; Heberman, 1981). The secretion by activated macrophages of soluble toxic factors, such as free oxygen radicals, has been implicated as a mechanism for the intraerythrocytic destruction of Babesia and Plasmodium (Clark, 1976; Clark, Willis, Richmond and Allison, 1977; Clark and Hunt, 1983). Macrophages can be activated by, and produce, interferon which stimulates lymphocytes, including NK cells (Nelson, 1980; Rönnblom, Ojo-Amaize, Franzén, Wigzell and Alm, 1983). NK cells, abundant in the spleen, may have a direct cytotoxic effect on parasitized cells or inhibit intracellular parasite development by the secretion of soluble factors (Allison, Christensen, Clark, Elford and Eugui, 1979; Clark, 1979; Eugui and Allison, 1980; Taverne, Dockrell and Playfair, 1982). The loss with splenectomy of a significant proportion of these interacting effector cell populations, as well as T-lymphocytes, could account for the recrudescence of the intraerythrocytic and intralymphocytic stages of T. annulata. Further studies may help to elucidate the interaction between humoral and cell-mediated immune mechanisms which interact to control Theileria infections.

CHAPTER SEVEN

IN VITRO CULTIVATION OF THEILERIA PARVA

7.1 Introduction

Intralymphocytic schizonts of Theileria parva are the most pathogenic stage of the parasite in the vertebrate host (Cowdry and Danks, 1933; De Kock, 1957; Barnett, 1960; De Martini and Moulton, 1973). Attention has, therefore, been focussed on cultivation of the macroschizont-infected lymphoid cells of T. parva, with comparatively little emphasis being placed on the intraerythrocytic stages of the parasite. Perhaps it is for this reason that there has been persistent disagreement as to whether T. parva piroplasms divide. The lack of conclusive evidence, from light and electron microscopic studies, to prove that intraerythrocytic multiplication occurred led a number of investigators to believe that T. parva piroplasms did not divide (Gonder, 1911a,b; Reichenow, 1940; Büttner, 1967a; Jarrett et al., 1969), while others considered limited multiplication possible (Koch, 1905; Nuttall et al., 1909; Cowdry and Danks, 1933; Neitz, 1964; Barnett, 1968).

The stationary erythrocyte culture system, in conjunction with electron microscopic studies, proved useful in elucidating the mode of intraerythrocytic multiplication of T. annulata (Chapters 3 and 5). Similar stationary cultures were established with blood from cattle infected with T. parva to see if the parasites would multiply in vitro (Section 7.2). Samples of infected blood and culture suspensions were subsequently examined by transmission electron microscopy to ascertain the ultrastructural features of the intraerythrocytic stages of T. parva (Section 7.3).

7.2 Establishment of Cultures

7.2.1 Experimental design: Blood was collected from calves 170 and 171 on days 13, 15 and 16 post infection with T. parva (Muguga). Suspensions of 3% (v/v) concentrated erythrocytes, obtained as described in Section 2.8.2, were prepared in complete medium consisting of 60% M199 with 40% foetal bovine serum. Cultures were established by depositing 1 ml aliquots of the suspensions into 2 cm^2 wells, which were gassed with 5% CO_2 in air and maintained as described in Section 2.8.3.

Cytocentrifuge smears were prepared from culture samples every 24 hours, stained with Giemsa and examined as described in Section 2.12.1. Evaluations were based on counts of the number of parasitized erythrocytes (PRBC) per 1000 erythrocytes and the number of parasites within 200 PRBC in smears prepared every two days from two wells for each factor.

Aliquots of suspensions prepared with blood collected on day 15 post infection were diluted 1:5 with 3% suspensions of unstained and fluorescein-stained normal bovine erythrocytes in M199/40 FBS on day 0 in vitro and evaluated for the invasion of erythrocytes by T. parva as described in Section 2.9.

7.2.2 Results: Parasite counts made from fresh blood smears on the day of culture establishment and samples taken at 48 hour intervals from the cultures are shown in Tables 7.1 and 7.2. Figures 7.1 and 7.2 graphically illustrate the relative change in the number of intraerythrocytic parasites in cultures established with blood collected from 170 and 171, respectively on day 13 post infection.

The incidence of multi-parasitized erythrocytes, most notably containing four parasites, increased during the cultivation period. Quadruplet forms, morphologically similar to the dividing forms seen in T. annulata cultures (Chapters 3-6), appeared in all of the T. parva cultures. Clusters of four small parasites with centrally oriented nuclei and basophilic cytoplasmic tails which radiated towards the periphery were seen in some erythrocytes (Figure 7.3a). Frequently parasites were seen to have separated from the cluster of four and dispersed within the host erythrocyte (Figures 7.3b and 7.4).

Quadruplet forms were rarely seen in the fresh blood smears prepared on day 13 post infection but by day 8 in vitro 10-25% of the parasitized erythrocytes contained these forms. Erythrocytes with more than four parasites contained at least one quadruplet form.

A small proportion, less than 0.5%, of the parasitized erythrocytes seen in the fresh blood smears prepared on days 15 and 16 post infection contained quadruplet forms. The incidence of erythrocytes with four parasites increased at least ten-fold during the subsequent 12 days of cultivation (Tables 7.1 and 7.2).

Extracellular parasites, often in large clusters, were seen in all of the cultures beginning on day 4 in vitro. Most of the extracellular parasites resembled, in size and appearance, the parasites in quadruplet forms. The number of parasitized erythrocytes in all of the cultures progressively decreased as the number of extracellular parasites increased over a 12 day period.

The parasitaemias were reduced below 2% in cultures diluted with suspensions of normal bovine erythrocytes, for the invasion assay,

Table 7.1 Theileria parva (Muguga) cultures established with blood collected from calf 170 on days 13, 15 and 16 post infection. Counts of parasitized erythrocytes (PRBC) arranged according to the number of parasites inside.

Sample	Day 13 post infection blood									Day 15 post infection blood									Day 16 post infection blood								
Day in vitro	Number of parasites in erythrocyte								PRBC per 1000 erythrocytes	Number of parasites in erythrocyte								PRBC per 1000 erythrocytes	Number of parasites in erythrocyte								PRBC per 1000 erythrocytes
	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8	
0 blood smear	195	4	1	0	0	0	0	0	19	194	4	0	2	0	0	0	0	66	181	15	2	2	0	0	0	0	103
	197	3	0	0	0	0	0	0	13	194	6	0	0	0	0	0	0	88	187	11	1	1	0	0	0	0	87
2	190	8	1	1	0	0	0	0	11	189	10	0	1	0	0	0	0	68	188	8	1	3	0	0	0	0	70
	195	4	0	1	0	0	0	0	14	196	3	0	1	0	0	0	0	52	185	7	1	7	0	0	0	0	76
4	188	9	3	0	0	0	0	0	9	179	8	1	12	0	0	0	0	41	184	8	1	4	3	0	0	0	71
	190	4	2	4	0	0	0	0	8	188	1	0	11	0	0	0	0	39	178	9	3	10	0	0	0	0	68
6	179	7	3	11	0	0	0	0	12	172	4	3	21	0	0	0	0	40	185	3	0	11	1	0	0	0	59
	186	4	0	10	0	0	0	0	6	178	2	0	19	1	0	0	0	46	174	8	1	14	1	1	0	1	51
8	168	6	4	22	0	0	0	0	7	166	8	2	23	1	0	0	0	48	177	5	1	16	1	0	0	0	45
	170	5	2	23	0	0	0	0	5	174	5	1	20	0	0	0	0	37	178	8	0	14	0	0	0	0	61
10	175	5	0	20	0	0	0	0	3	173	7	1	19	0	0	0	0	24	159	16	4	20	0	0	0	1	35
	175	6	0	19	0	0	0	0	2	173	6	1	20	0	0	0	0	26	167	12	3	15	1	2	0	0	42
12	190	5	0	5	0	0	0	0	3	180	4	0	16	0	0	0	0	18	174	9	5	10	2	0	0	0	30
	177	6	0	16	1	0	0	0	3	182	7	1	10	0	0	0	0	14	179	4	2	15	0	0	0	0	23

Figures for each day are counts of parasites in 200 PRBC and number of PRBC per 1000 erythrocytes in samples from two cultures.

Table 7.2 Theileria parva (Muguga) cultures established with blood collected from calf 171 on days 13, 15 and 16 post infection. Counts of parasitized erythrocytes (PRBC) arranged according to the number of parasites inside.

Sample Day in vitro	Day 13 post infection blood: Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	PRBC per 1000 erythrocytes	Day 15 post infection blood: Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	PRBC per 1000 erythrocytes	Day 16 post infection blood: Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	PRBC per 1000 erythrocytes
0	187	8	5	0	0	0	0	0	7	193	4	0	3	0	0	0	0	92	183	14	2	1	0	0	0	0	145
	189	6	4	1	0	0	0	0	8	188	10	0	2	0	0	0	0	91	192	6	1	1	0	0	0	0	158
2	188	3	4	5	0	0	0	0	8	186	8	1	5	0	0	0	0	63	184	9	3	4	0	0	0	0	113
	192	7	0	1	0	0	0	0	19	184	11	1	3	1	0	0	0	70	188	7	0	5	0	0	0	0	149
4	191	6	1	2	0	0	0	0	9	174	3	1	21	1	0	0	0	58	189	3	0	8	0	0	0	0	122
	190	7	0	3	0	0	0	0	6	170	4	1	25	0	0	0	0	53	171	6	2	19	2	0	0	0	120
6	176	6	1	17	0	0	0	0	11	182	7	1	9	1	0	0	0	51	175	7	0	17	0	0	0	1	92
	176	10	2	11	0	0	0	1	6	176	9	2	13	0	0	0	0	50	172	8	0	20	0	0	0	0	101
8	152	2	3	43	0	0	0	0	6	167	3	1	28	1	0	0	0	52	163	12	0	24	1	0	0	0	82
	153	9	1	37	0	0	0	0	5	162	4	2	31	1	0	0	0	44	171	4	0	24	0	0	1	0	85
10	144	6	0	48	1	0	0	1	4	165	8	1	24	0	0	0	2	31	161	10	4	24	1	0	0	0	101
	145	3	0	51	1	0	0	0	3	168	7	0	25	0	0	0	0	39	152	12	3	31	1	1	0	0	86
12	163	11	0	26	0	0	0	0	3	161	3	3	33	0	0	0	0	13	173	4	2	18	3	0	0	0	74
	156	5	2	37	0	0	0	0	5	167	10	3	18	2	0	0	0	29	179	6	1	14	0	0	0	0	58

Figures for each day are counts of parasites in 200 PRBC and number of PRBC per 1000 erythrocytes in samples from two cultures.

Figure 7.1 Theileria parva (Muguga) cultures established with blood from calf 170 collected on day 13 post infection.
Figures represent mean number of parasitized erythrocytes per 100 counted in samples from 2 cultures with specified number of parasites inside.

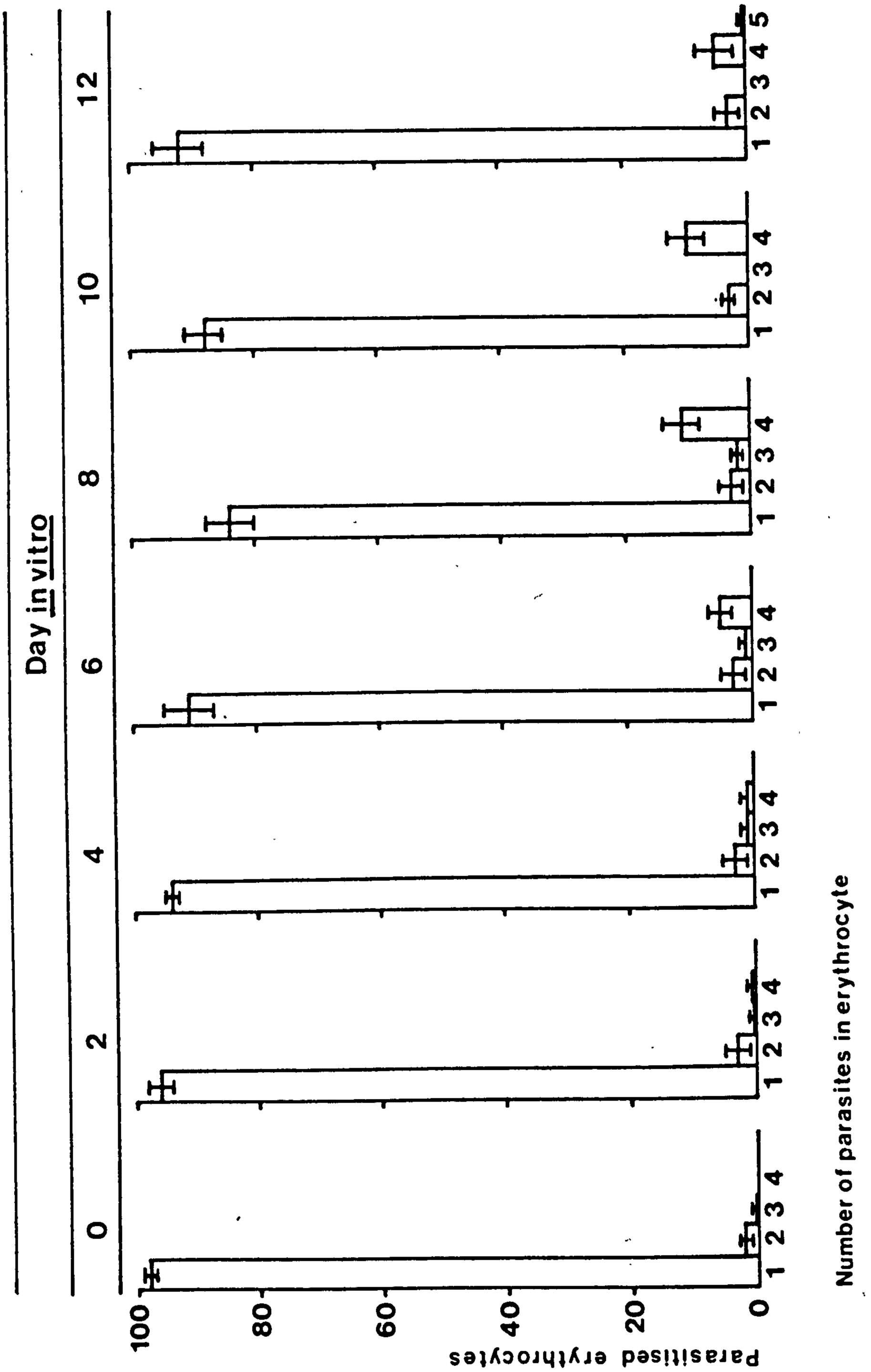

Parasitised erythrocytes
100
80
60
40
20
0
Day in vitro
0
2
4
6
8
10
12
Number of parasites in erythrocyte
1 2 3 4 5

Figure 7.2 Theileria parva (Muguga) cultures established with blood from calf 171 collected on day 13 post infection.
Figures represent mean number of parasitized erythrocytes per 100 counted in samples from 2 cultures with specified number of parasites inside.

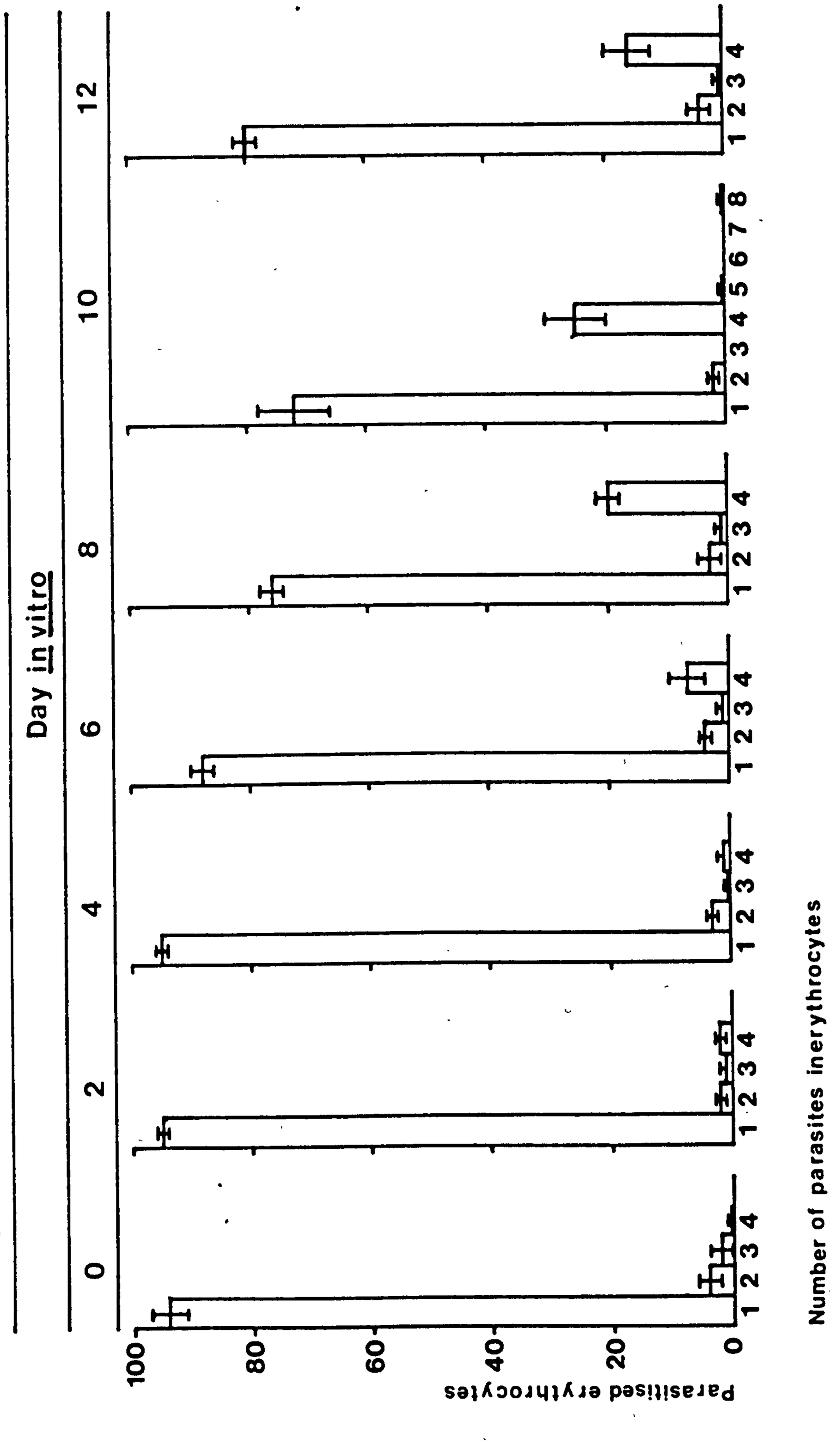
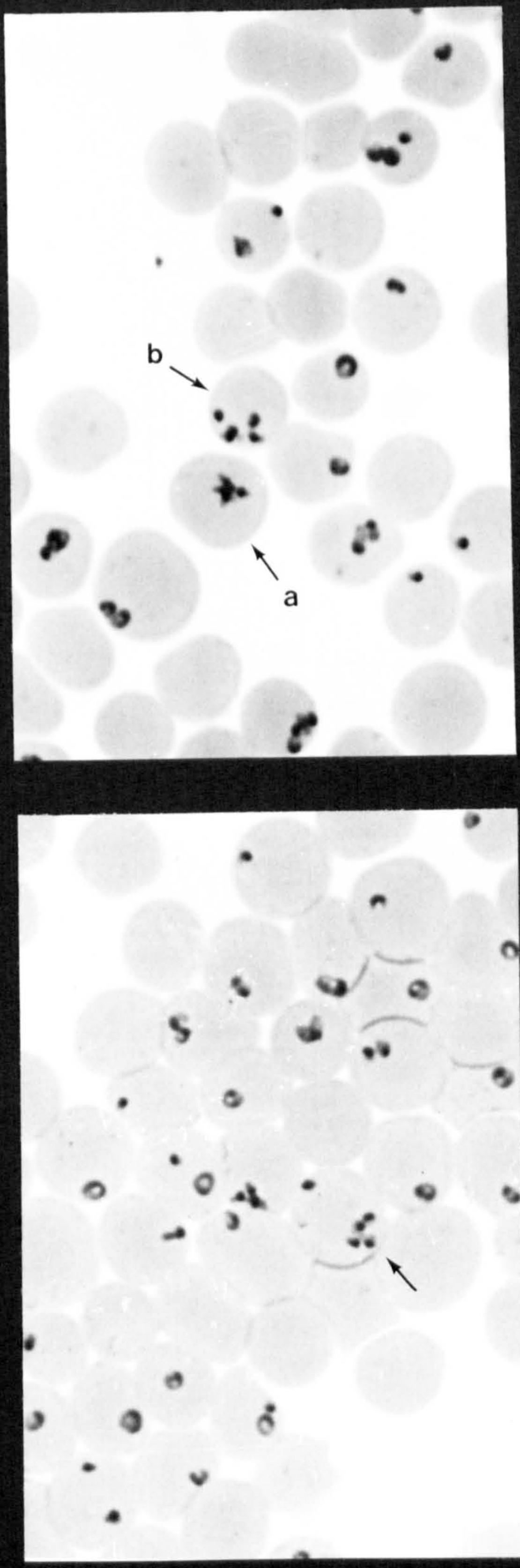

Parasitised erythrocytes
100
80
60
40
20
0
Day in vitro
0
2
4
6
8
10
12
Number of parasites inerythrocytes
1 2 3 4
1 2 3 4 5 6 7 8

Figure 7.3

<u>Theileria parva</u> (Muguga) after 2 days in stationary erythrocyte cultures established with blood from calf 171, collected on day 20 post infections, are shown in Figures 7.3 and 7.4. Arrows indicate quadruplet configurations of parasites.

Figure 7.4


b
a

and did not increase during the subsequent six days *in vitro* (Table 7.3). The morphology and number of intraerythrocytic parasites were similar in cultures receiving suspensions of fluorescein-stained erythrocytes and in cultures diluted with unstained erythrocytes. Extracellular parasites were observed in both sets of cultures. Examination of cultures with fluorescein-stained erythrocytes revealed parasites only within the original unstained erythrocytes from the donor calf.

7.3 Electron Microscopic Evaluation

7.3.1 *Experimental design*: The protocol for sample fixation and section preparation was as described in Section 2.12.2. Giemsa stained cytocentrifuge smears were prepared from aliquots of each sample processed for transmission electron microscopy (TEM).

A lymph node biopsy sample was taken from calf 170 on day 19 post infection with a 14 gauge 1½ inch needle and expelled directly into a solution of 2.5% glutaraldehyde fixative. The sample was fixed for two hours in the glutaraldehyde solution before being processed for TEM (Section 2.12.2).

Aliquots of defibrinated blood taken from calves 170 and 171 on days 18-20 post infection with *T. parva* (Muguga) were used to prepare suspensions for piroplasm cultures or were fixed within 30 minutes of collection for TEM. The culture suspensions, prepared as described in Section 7.2.1, were deposited in 3 ml aliquots into vertical flasks, gassed with 5% CO_2 in air and maintained as described in Section 2.8.3.

Samples of the defibrinated blood and of culture suspensions after 2-4 days *in vitro* were fixed and processed for TEM as described in Section 2.12.2.

Table 7.3 Theileria parva (Muguga) cultures established with blood collected from calves 170 and 171 on day 15 post infection: Diluted 1:5 with suspensions of unstained (US) and fluorescein-stained (FS) erythrocytes

Culture sampled		Calf 170						Calf 171					
Day in vitro	Erythrocyte suspension added to culture	Number of parasites in erythrocyte					PRBC per 1000 erythrocytes	Number of parasites in erythrocyte					PRBC per 1000 erythrocytes
		1	2	3	4	5		1	2	3	4	5	
0	FS	98	2	0	0	0	10	96	3	1	0	0	14
	US	96	4	0	0	0	9	94	4	1	1	0	13
2	FS	95	2	0	3	0	10	84	10	2	4	0	8
	US	95	3	0	2	0	13	88	9	2	1	0	12
6	FS	83	4	0	13	0	8	87	2	1	9	1	7
	US	85	3	1	11	0	5	83	6	1	10	0	8

Figures are mean counts of parasites in 200 parasitized erythrocytes (PRBC) and number of PRBC per 1000 erythrocytes in samples from two cultures.

7.3.2 Results:

(a) T. parva in lymph node and blood samples - The ultrastructural features of the exoerythrocytic (intralymphocytic) merozoites of T. parva are shown in the electron micrographs in Figures 7.5 and 7.6 taken of the lymph node biopsy sample from calf 170.

Merozoite anlagen are seen beneath the plasmalemma of the T. parva schizont in a section through an infected lymphoid cell (Figure 7.5). The appearance of inner membrane segments and electron dense rhoptries mark the sites of merozoite formation in the schizont.

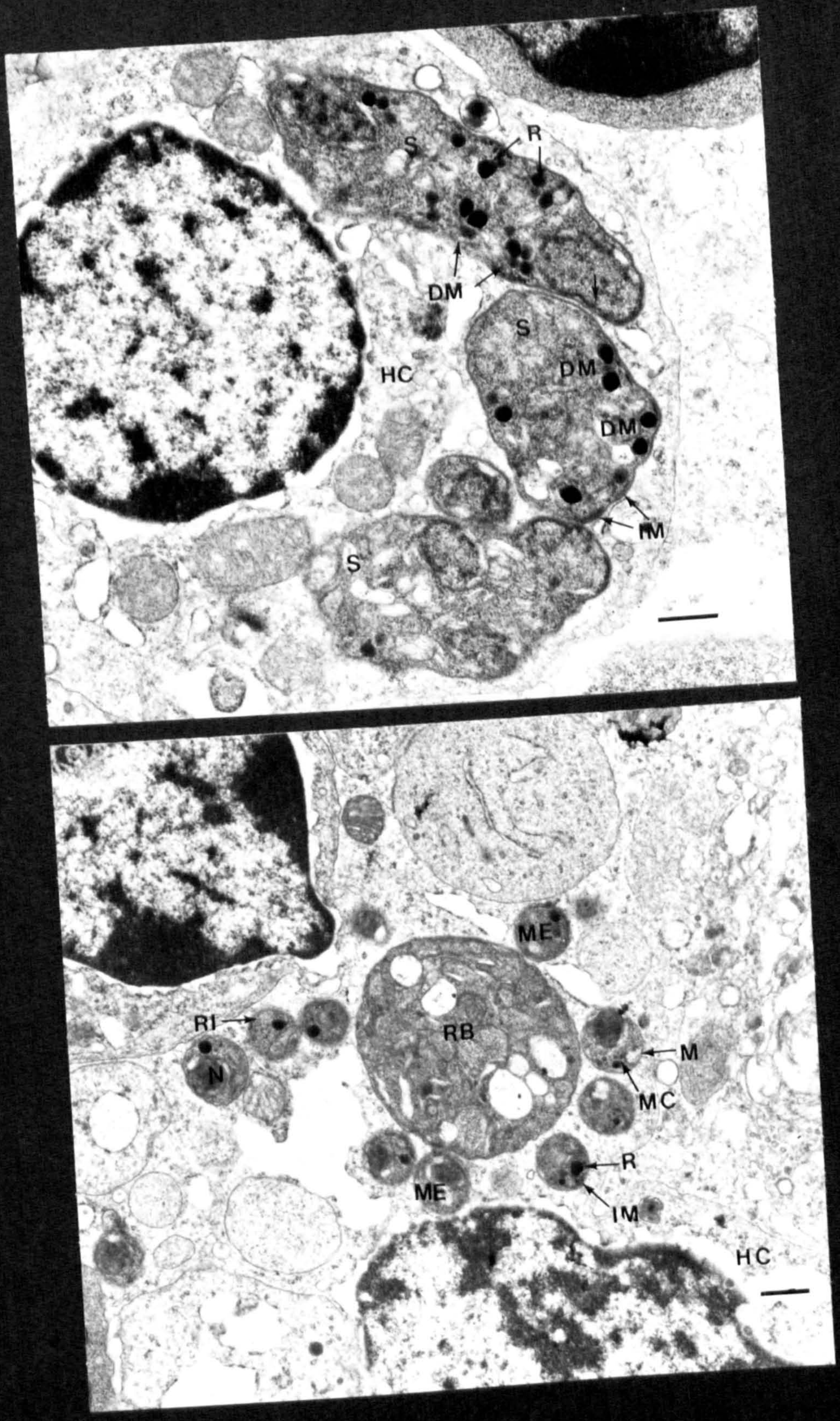
Exoerythrocytic merozoites of T. parva have a nucleus with homogenous karyoplasm, a double membraned organelle (mitochondrion?) and numerous free cytoplasmic ribosomes (Figure 7.6). The characteristic structures of an apical complex, consisting of an inner membrane segment, rhoptries and micronemes are seen in the exoerythrocytic merozoites.

The electron micrograph in Figure 7.7 was taken of a defibrinated blood sample from calf 171 collected on day 20 post infection, when there was a 48% parasitaemia. T. parva piroplasms, situated directly in the cytoplasm of the host erythrocyte, had a single limiting plasmalemmal membrane and a prominent nucleus with homogenous karyoplasm. The cytoplasm contained numerous free ribosomes, and two double-membraned organelles with osmiophilic, filamentous strands projecting from the inner membrane into the lumen. A cytostome and single food vacuole, containing material which was identical in appearance to the cytoplasm of the host erythrocyte, were seen in some parasites.

Parasites with similar ultrastructural features were observed in the erythrocytes of both calves in blood and culture samples.

Figure 7.5 Section through a lymphoid cell (HC) with schizonts (S) of Theileria parva (Muguga) in a lymph node biopsy sample from calf 170. The sites of merozoite formation (DM) are marked by the presence of inner membrane segments (IM), and electron dense rhoptries (R) beneath the plasmalemma of the schizonts.
Bar = 0.5μ (x 18750)

Figure 7.6 Exoerythrocytic merozoites (ME) associated with a schizont residual body (RB) are being released from an infected lymphoid cell (HC). Differentiated merozoites have a nucleus (N), free cytoplasmic ribosomes (RI), double membraned organelles (M), segments of inner membranes (IM), rhoptries (R) and micronemes (MC).
Bar = 0.5μ (x 15000)


S
R
DM
S
HC
DM
DM
IM
S
RI
N
RB
ME
M
MC
R
ME
IM
HC

Figures 7.8-7.10 were taken of T. parva after two days in stationary erythrocyte cultures established with blood collected from calf 171 on day 20 post infection. The appearance of clusters of the intra-erythrocytic parasites are shown in the light micrographs taken of Giemsa stained cytocentrifuge smears prepared with the culture samples prior to fixation for TEM (Figures 7.3 and 7.4).

Segments of inner membranes and electron dense rhoptries were seen beneath the plasmalemma of a proportion of the cultivated intra-erythrocytic parasites (Figure 7.8). These apical complex structures marked the sites of merozoite anlagen. A maximum of four developing merozoites were seen within the plasmalemma of a single intraerythro-cytic parasite (Figure 7.9). Developing merozoites had distinct nuclei with homogenous karyoplasm, numerous free cytoplasmic ribo-somes, apical segments of inner membranes, rhoptries and micronemes. The electron dense rhoptries ranged in size from 84-170 nm while similar structures not exceeding 35 nm had the appearance of micro-nemes. Double membraned organelles (mitochondria?) are not shown in the electron micrographs presented in this section but these structures were seen individually, in the cytoplasm of some intra-erythrocytic T. parva merozoites in vitro. The double membraned organelles were 80-280 nm in size and had filamentous strands pro-jecting from the inner membrane into the lumen. Differentiated merozoites had a spherical or pyriform shape and ranged in size from 0.4-0.6 μ. Merozoites in the process of division had intact nuclear membranes even when they were still attached to a residual body (Figure 7.10).

Figure 7.7 Section through a Theileria parva (Muguga) piroplasm in an erythrocyte (HC) of calf 171, taken from a defibrinated blood sample collected day 20 post infection. Note the single plasmalemmal membrane (PM), nucleus (N), double membraned organelles (M), free cytoplasmic ribosomes (RI) and cytostome (C).
Bar = 0.5μ (x 53250)

Figures 7.8-7.10 are of T. parva after 2 days in stationary erythrocyte cultures established with blood from calf 171 collected on day 20 post infection

Figure 7.8 Three sites of merozoite formation (IM) within the parasite are marked by the presence of inner membrane segments (IM) and electron dense rhoptries (R).
Bar = 0.5μ (x 53250)

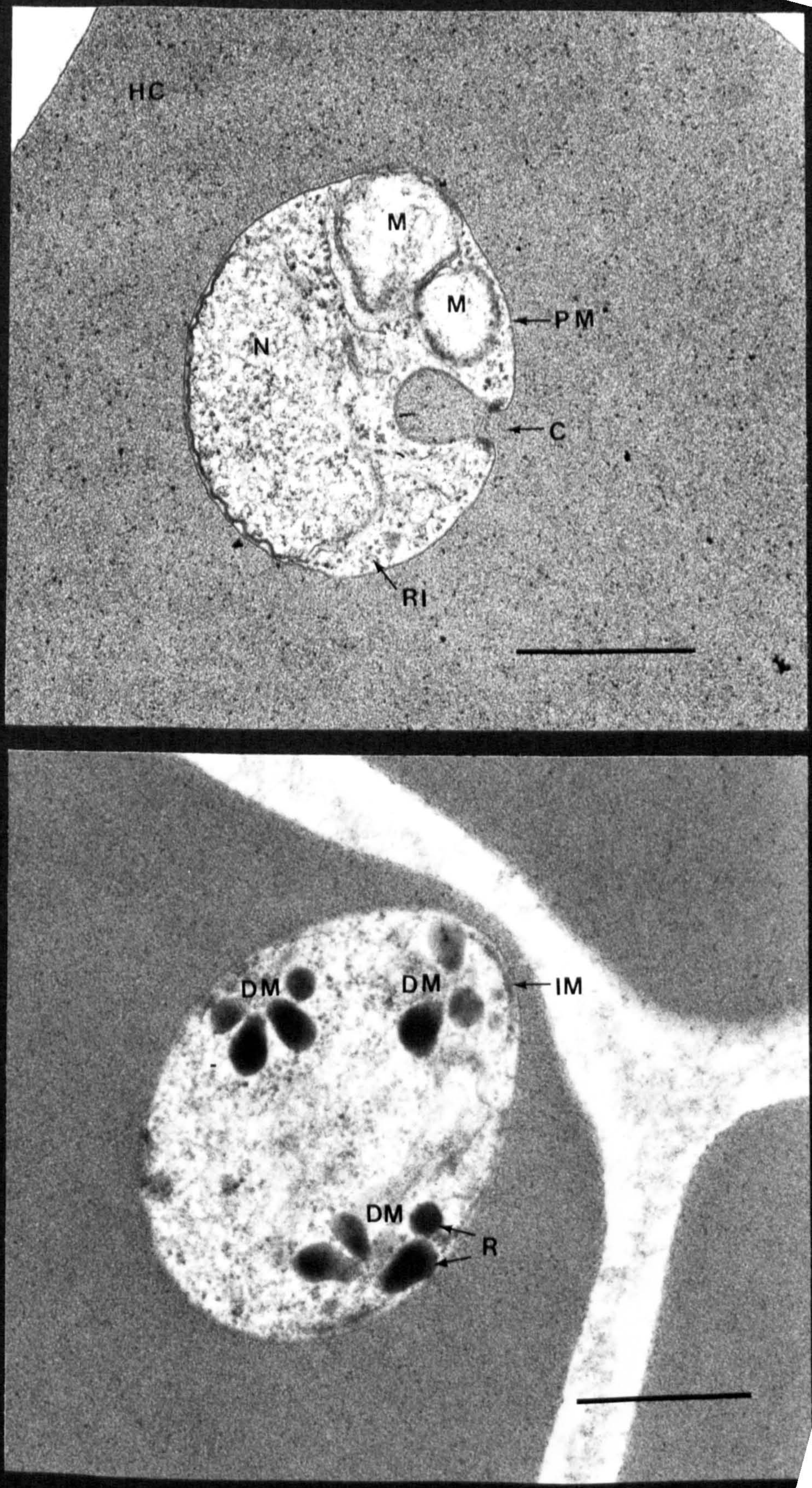

HC
M
M
PM
N
C
RI
DM
DM
IM
DM
R

Figure 7.9 T. parva in vitro showing four developing merozoites within a single plasmalemmal membrane.
Bar = 0.5μ (x 53250)

Figure 7.9(i) Diagrammatic representation of parasite in Figure 7.9 illustrating the four nuclei (N) of the merozoite anlagen, double membraned organelles (M), inner membrane segments (IM), rhoptries (R) and micronemes (MC).


DM
DM
DM
DM

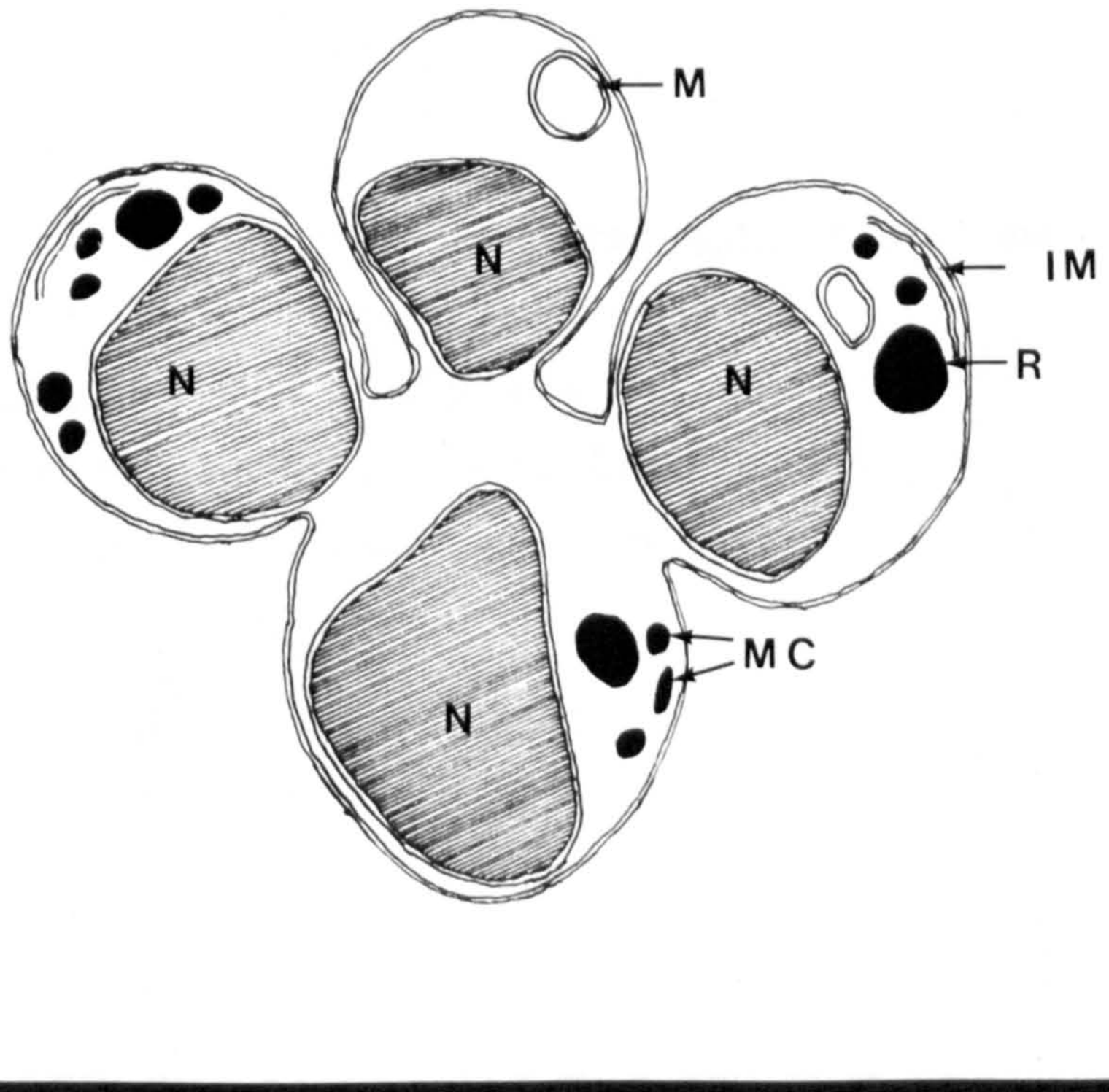
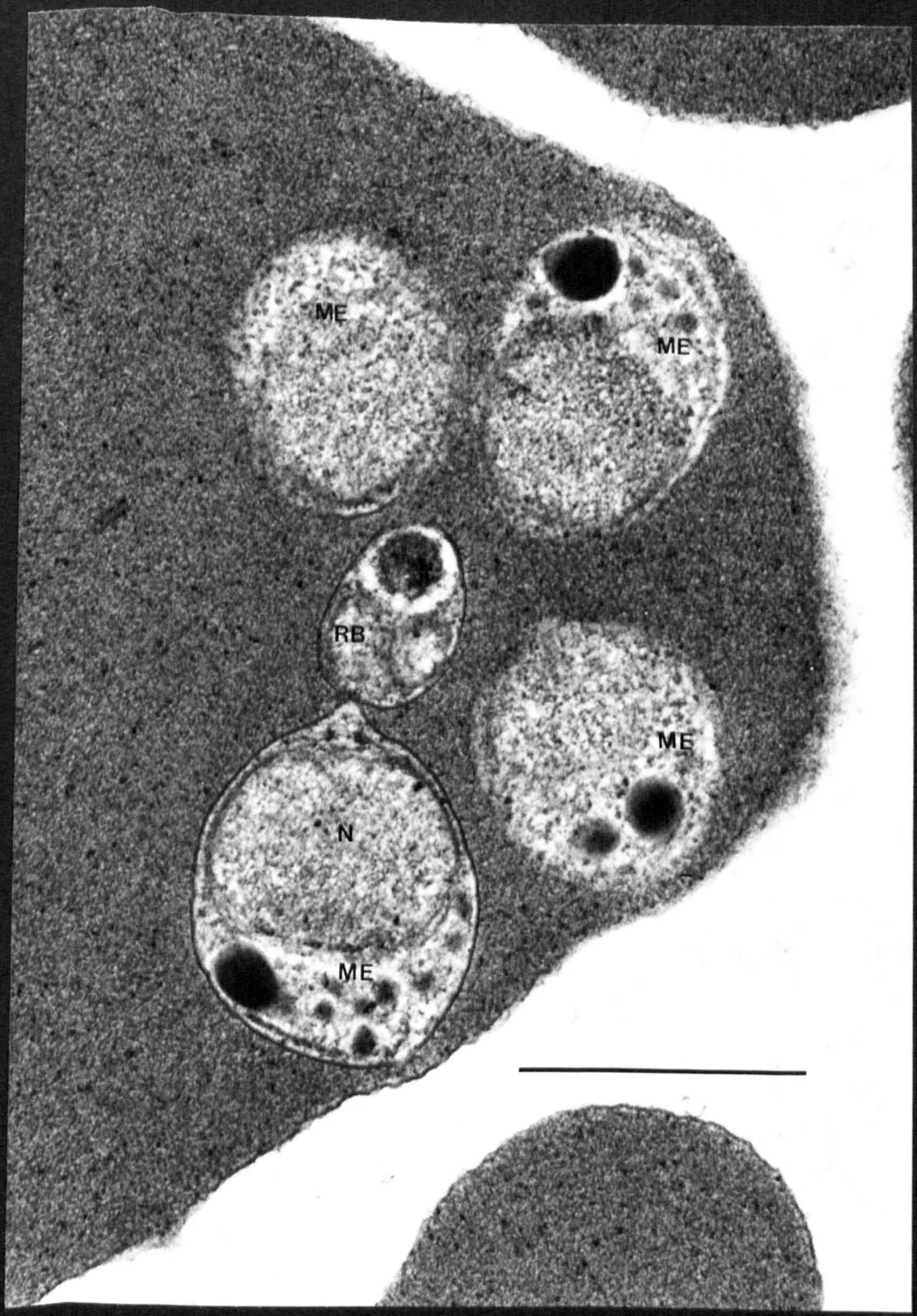

M
N
N
N
N
IM
R
MC

Figure 7.10 Four merozoites (ME) of T. parva in vitro separating from a residual body (RB). The merozoite still attached to the residual body has a distinct nucleus (N) surrounded by an intact nuclear membrane.

Bar = 0.5μ (x 88750)


ME
ME
RB
ME
N
ME

7.4 Discussion

The incidence of erythrocytes with four parasites increased in all of the stationary erythrocyte cultures established with blood from T. parva-infected calves 170 (Table 7.1, Figure 7.1) and 171 (Table 7.2, Figure 7.2). The four small parasites in clusters or dispersed within the erythrocyte (Figures 7.3 and 7.4) were morphologically similar to the intraerythrocytic merozoites of T. annulata (Figures 3.1 and 3.2). T. parva appeared to be dividing in vitro into a maximum of four daughter parasites within infected erythrocytes.

There was an increased incidence of erythrocytes with more than four parasites in some cultures. These cells invariably included at least one quadruplet form. Apparently one of the piroplasms in these multi-parasitized erythrocytes had divided during the cultivation period while the accompanying piroplasms remained unchanged.

In T. annulata cultures merozoites appeared to be unable to leave the host erythrocytes after division. The accumulation of extracellular parasites in T. parva cultures indicated that parasites were leaving the erythrocytes after multiplication but were not reinvading in appreciable numbers. Several observations supported this supposition. The extracellular forms in T. parva cultures were similar in size and morphology to the small intraerythrocytic parasites in clusters of four. The appearance of these extracellular parasites coincided with the apparent increase in the number of dividing intraerythrocytic parasites (i.e. quadruplet forms) in the same cultures. The number of parasitized erythrocytes gradually decreased in all of the cultures over a 12 day period as more parasites accumulated extracellularly in the medium (Tables 7.1 and 7.2). The accumulation of

extracellular parasites indicated that the number of parasites dividing may have been much greater than the number of quadruplet forms counted.

Quadruplet forms and extracellular parasites were seen in cultures diluted with unstained and fluorescein-stained normal bovine erythrocytes. However, during a six day observation period, the parasitaemias did not increase in these cultures (Table 7.3) and parasites were never seen to have invaded fluorescein-stained erythrocytes.

Exoerythrocytic merozoites form by a process of schizogony in T. parva-infected lymphoid cells. Electron micrographic studies have shown that the formation of merozoites begins with the appearance of subplasmalemmal membrane segments and rhoptries (Figure 7.5) within the schizont (Jarrett and Brocklesby, 1966; Mugera and Munyua, 1973; Schein et al., 1978). Exoerythrocytic merozoites bud from the schizont and are released from the host lymphoid cell, often in association with a residual body of the schizont (Figure 7.6). After invading erythrocytes these merozoites lose their apical complex structures, consisting of the rhoptries and inner membrane, and assume the appearance of piroplasms (Büttner, 1967a).

The cytoplasmic organelles of the intraerythrocytic piroplasms included two double-membraned organelles which are propounded to be acristate mitochondria (Aikawa, 1971; Rudzinska and Trager, 1977; Fawcett et al., 1982; Weber, 1982). The presence of a cytostome (Figure 7.5) and food vacuoles, containing erythrocytic cytoplasm, in some piroplasms suggests that this is a feeding stage for the parasite. The term, trophozoite, seems therefore appropriate to describe this developmental stage of T. parva.

Intraerythrocytic multiplication as observed in stationary erythrocyte cultures has not been previously described in electron microscopic studies of T. parva. The formation of merozoites from T. parva piroplasms in vitro began with the appearance of inner segments of double membranes and rhoptries beneath the plasmalemma (Figure 7.8). Nuclear division apparently preceded cytoplasmic division as the nuclei of budding merozoites were distinct and surrounded by intact membranes (Figures 7.9 and 7.10). The mode of intraerythrocytic multiplication was by schizogony resulting in the formation of a maximum of four merozoites.

The number of merozoites which bud from the intralymphocytic schizonts of T. parva are greater, but the schizogonous process of merozoite formation, is in many respects, similar to that observed in the electron micrographs of the cultivated intraerythrocytic parasites. The exoerythrocytic merozoites produced from microschizonts in T. parva-infected cattle (Figure 7.6) have the same ultrastructural features as the intraerythrocytic merozoites observed in stationary cultures (Jarrett and Brocklesby, 1966; Büttner, 1967a,b; Mugera and Munyua, 1973; Schein et al., 1978).

Clusters of four small parasites like those seen in vitro have been observed by the author and others in the blood of cattle infected with T. parva (D.W. Brocklesby and C.G.D. Brown, personal communication; A.D. Irvin, personal communication; see Figures 1.2 and 1.3). Divergent opinions have been put forth regarding the significance of quadruplet or cross forms of T. parva. Early investigators proposed that these forms represented a primary mode of intraerythrocytic multiplication (Koch, 1905; Bettencourt et al., 1907). However, lack of conclusive

evidence to support this contention resulted in speculation that piroplasms of T. parva may not divide (Wenyon, 1926; Reichenow, 1940; Jarrett et al., 1969) or that division was by binary fission (Cowdry and Ham, 1932; Cowdry and Danks, 1933).

Gonder (1910, 1911a,b) proposed that quadruplet forms were sexual stages of T. parva. Recent studies suggest, however, that large spherical macrogametes and spindle-shaped microgametes, with spiky processes, are the sexual forms of T. parva which develop in the gut lumen of the infected tick (Mehlhorn and Schein, 1976; Schein et al., 1977). Parasites resembling these sexual forms were not observed in stationary erythrocyte cultures maintained at 37°C.

The stationary erythrocyte culture system tested was not adequate to sustain continuous growth and multiplication of T. parva. Nonetheless, the system proved useful in isolating the parasite so that the mode of intraerythrocytic multiplication could be studied, without the interference of exoerythrocytic merozoites invading erythrocytes or the immunological response of the host. The culture system supported and may have served to stimulate a sufficient number of T. parva piroplasms to multiply in a relatively short period of time so that electron micrographic studies, which illustrated the mode of intraerythrocytic multiplication, could be conducted. These studies showed that T. parva is capable of intraerythrocytic multiplication by a schizogonous process.

The fact that merozoites were capable of leaving the erythrocytes in T. parva cultures suggested that with further work, a system may be developed which would sustain merozoite infectivity in vitro. Studies on the effect of immune factors on theilerial merozoites or on the

infectivity of merozoites for different cell types could then be conducted. The antigenic relationship between sporozoites, exoerythrocytic merozoites in lymphoid cell cultures and merozoites isolated from stationary erythrocyte cultures could be investigated. These three extracellular stages of Theileria would, theoretically, be the most susceptible to destruction by the host's immune response, and might have common antigenic determinants.

Further studies on the intraerythrocytic stages of T. parva are required, particularly in the light of observations reported in this chapter. Although the piroplasms of T. parva have little pathological effect on the host, they are still important as this is the parasite stage which is infective for ticks (Nuttall, 1914; Cowdry and Danks, 1933; Wilde, 1967; Irvin and Young, 1980). The potential problem of a persistent carrier state in T. parva-infected cattle has been recently acknowledged (Dolan, 1981; Young, Leitch and Newson, 1981; Chema and Brocklesby, 1981). Intraerythrocytic multiplication may play a significant role in propagation of the parasite in chronic carrier cattle which have recovered from clinical East Coast fever, and remain infective for ticks.

CHAPTER EIGHT

IN VITRO CULTIVATION OF BABESIA BOVIS

The potential applications of the Theileria annulata and T. parva piroplasm cultures were limited by the inability to maintain parasite growth for prolonged periods in vitro. Stationary erythrocyte cultures of Babesia bovis, by contrast, support continuous propagation and provide a readily available source of parasites for experiments (Levy and Ristic, 1980). Studies on the incorporation of tritiated nucleic acid precursors, described in this chapter, were accordingly conducted with cultures of two strains of B. bovis established with blood from infected cattle.

8.1 Establishment of B. bovis Cultures

8.1.1 Introduction: The series of experiments described in this chapter utilized B. bovis grown continuously in microaerophilous stationary phase cultures (Levy and Ristic, 1980). Cultures were established with blood from cattle which had been infected with either a culture-derived stabilate of a Mexican isolate (Smith et al., 1978) or a blood stabilate originating from an infected calf in South Africa (Taylor and McHardy, 1979). Bovine aortic endothelial (BAE) monolayers were employed as feeder layers in some cultures during the initial isolation in an attempt to overcome the minimum seeding density requirement of B. bovis (Levy and Ristic, 1980).

8.1.2 Materials and methods: Calves 198 and 199 were splenectomized (Section 2.3) and infected, respectively, with the South African and Mexican isolates of B. bovis as described in Section 2.6.

Blood was collected daily from the time when the parasitaemia exceeded 0.01% and used to prepare suspensions of 10% (v/v) concentrated erythrocytes, obtained as in Section 2.8.2, in complete medium consisting of 60% Medium 199 and 40% normal adult bovine serum (NBS). The complete medium was routinely supplemented as described in Section 2.7.1.

Cultures were initially established by depositing 0.2 ml aliquots of the 10% suspensions into replicate microtitre wells, some of which were preseeded with BAE monolayers (Section 2.8.1). Daily isolations were made in wells on separate microtest plates and placed in separate culture incubation boxes for the two parasites. The boxes were gassed with 5% CO_2 and air, and maintained as described in Section 2.8.3.

At 48-72 hour intervals, when the parasitaemia exceeded 12-15%, the culture suspensions were diluted with fresh 10% (v/v) suspensions of concentrated normal bovine erythrocytes, obtained as in Section 2.8.2, in the same medium and passaged into 2 cm^2 wells or flasks. The seeding density and total culture depth after daily medium changes, in routine maintenance, are shown below.

Vessel	Culture surface area (cm^2)	Culture volume	Volume replaced with medium change (ml)
Microtitre wells	0.32	0.2	0.2
16 mm diameter wells	2	1.25	1
Flasks - vertical	7	5	4
horizontal	25	15	7-10

8.1.3 Results: The isolations made from each calf are summarised in Table 8.1. Cultures were successfully established from both calves on consecutive days when the parasitaemias ranged from less than 0.1% to a maximum of 3% for the South African strain and 15% for the Mexican strain. The first isolation from the calf infected with B. bovis (Mexico), when the parasitaemia was less than 0.1%, was only successful in cultures with a BAE monolayer. In the corresponding cultures without a monolayer the parasitaemia remained below 1% and gradually decreased within six days.

8.2 Complex Media and Serum Concentrations Tested in Mexican Strain Cultures

8.2.1 Introduction: The complete medium recommended for B. bovis cultures consists of Medium 199 with a high concentration of NBS (Erp et al., 1980; Levy and Ristic, 1980). The purine and pyrimidine bases in M199 could competitively inhibit the incorporation of tritiated compounds (Trager, 1971; Tracy and Sherman, 1972; Chulay, Haynes and Diggs, 1983). Preliminary experiments were, therefore, conducted with B. bovis (Mexico) in an attempt to reduce the serum concentration and identify a complex medium capable of maintaining parasite growth, which could be used in radioisotope incorporation experiments.

8.2.2 Materials and methods: Factors tested in this experiment were:

(a) Concentrations of NBS - compared as a percentage of the complete medium with M199

(i) 10%
(ii) 20%
(iii) 40%

Table 8.1 Establishment of Babesia bovis cultures with blood from calves (198, 199) infected with the South African and Mexican strains

Day post infection	South African strain Calf 198			Mexican strain Calf 199		
	% parasitaemia	Isolation without BAE	Isolation with BAE	% parasitaemia	Isolation without BAE	Isolation with BAE
5	<0.01	NA	NA	<0.1	-	+
6	<0.01	NA	NA	0.1	+	+
7	<0.1	+	+	1.4	+	+
8	1.0	+	+	15.0	+	+
9	3.0	+	+	Death		
10	babesiacide treatment	NA	NA			

NA = Not attempted

\- = cultures not established with isolate

\+ = successful establishment of cultures, B. bovis growth maintained with repeated passage

(b) Complex media - compared as 80% of the complete medium with 20% NBS

(i) M199

(ii) MEM-H (Hanks' salts)

(iii) MEM-E (Earle's salts)

(iv) RPMI 1640

(v) L-15

In each experiment a 10 ml aliquot of B. bovis (Mexico) culture suspension, with a parasitaemia of 8-12%, was centrifuged at 1000 x g for ten minutes. The concentrated erythrocytes were used to prepare 10% (v/v) suspensions in each of the complete test media. The culture suspensions were diluted with 10% (v/v) suspensions of concentrated normal bovine erythrocytes, obtained as in Section 2.8.2, in the same complete medium, to reduce the parasitaemia to 0.5-1.0%.

Cultures were evaluated by counting the number of parasitized erythrocytes per 1000 erythrocytes in one Giemsa stained cytocentrifuge smear from each of two wells in the serum concentration comparison and three wells in the media comparison. Each well was sampled once only. The erythrocyte counts were based on estimates. To assess the accuracy of the estimates random counts were made of 12 fields, viewed under oil at x 1000 magnification during the evaluations and compared to the estimated counts.

8.2.3 Results: The multiplication rates of B. bovis (Mexico) in complete medium with NBS concentrations of 10, 20 or 40% are illustrated graphically in Figure 8.1. Growth was sustained with repeated passages in M199 with either 20% or 40% NBS, but not when the serum concentration was reduced to 10%. In M199/20 NBS or M199/40 NBS the parasitaemias generally reached peak levels of 12-16% and then declined.

Figure 8.1 Growth of Babesia bovis (Mexico) in complete medium with M199 and either 10, 20 or 40% normal adult bovine serum (NBS).
Figures are means ± standard deviations of counts from 2 cultures.

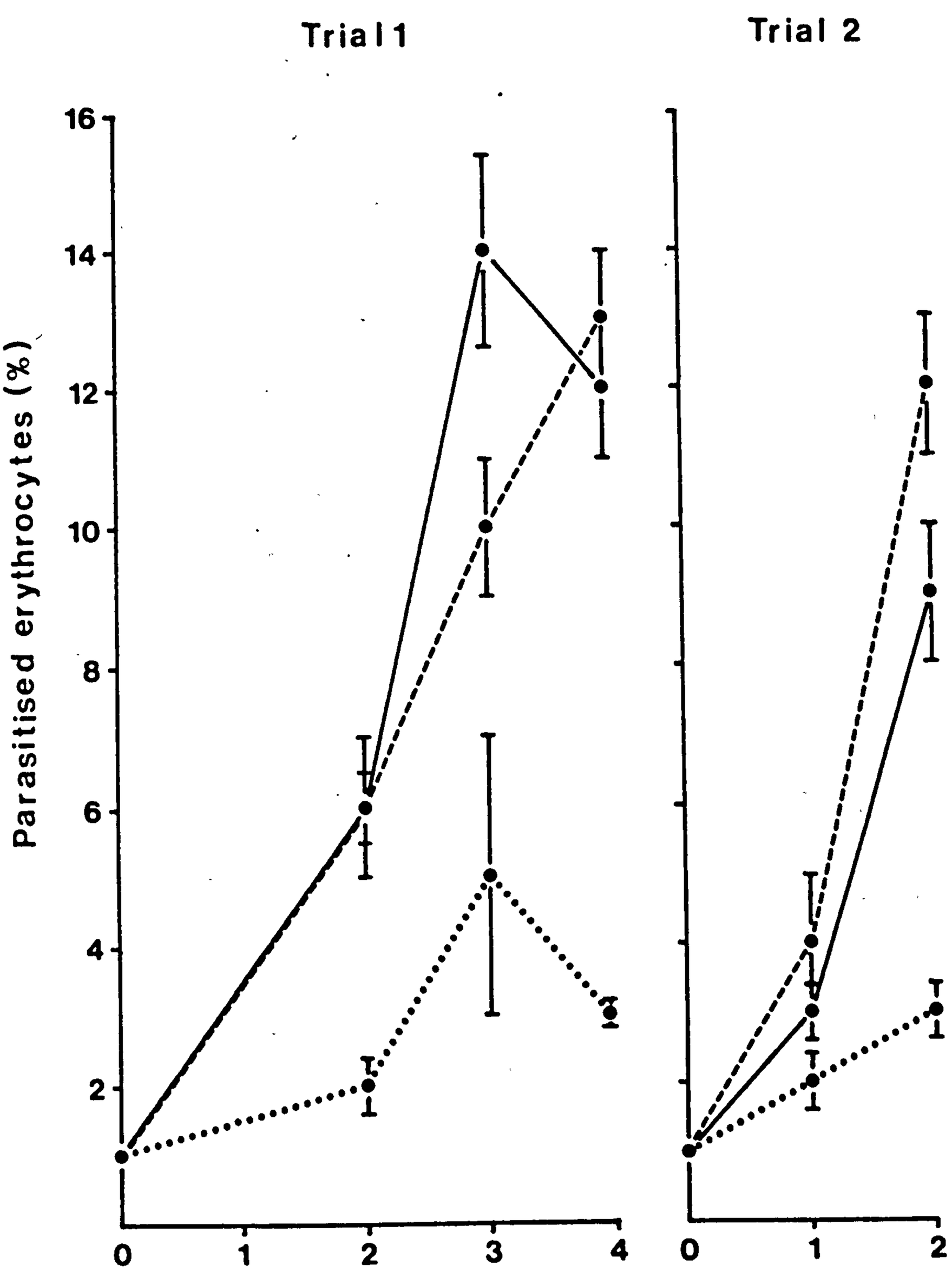

Trial 1
Trial 2
Parasitised erythrocytes (%)
16
14
12
10
8
6
4
2
0
1
2
3
4
0
1
2
Day in vitro
Concentration of NBS
10%
20%
40%

Figure 8.2 shows the relative increase in the number of parasitized erythrocytes (% parasitaemia) maintained over two passages in M199/20 NBS, MEM-H/20 NBS and MEM-E/20 NBS, as compared to M199/40 NBS. The peak number of parasites was greatest in the cultures with M199 but multiplication was maintained in MEM-H/20 NBS.

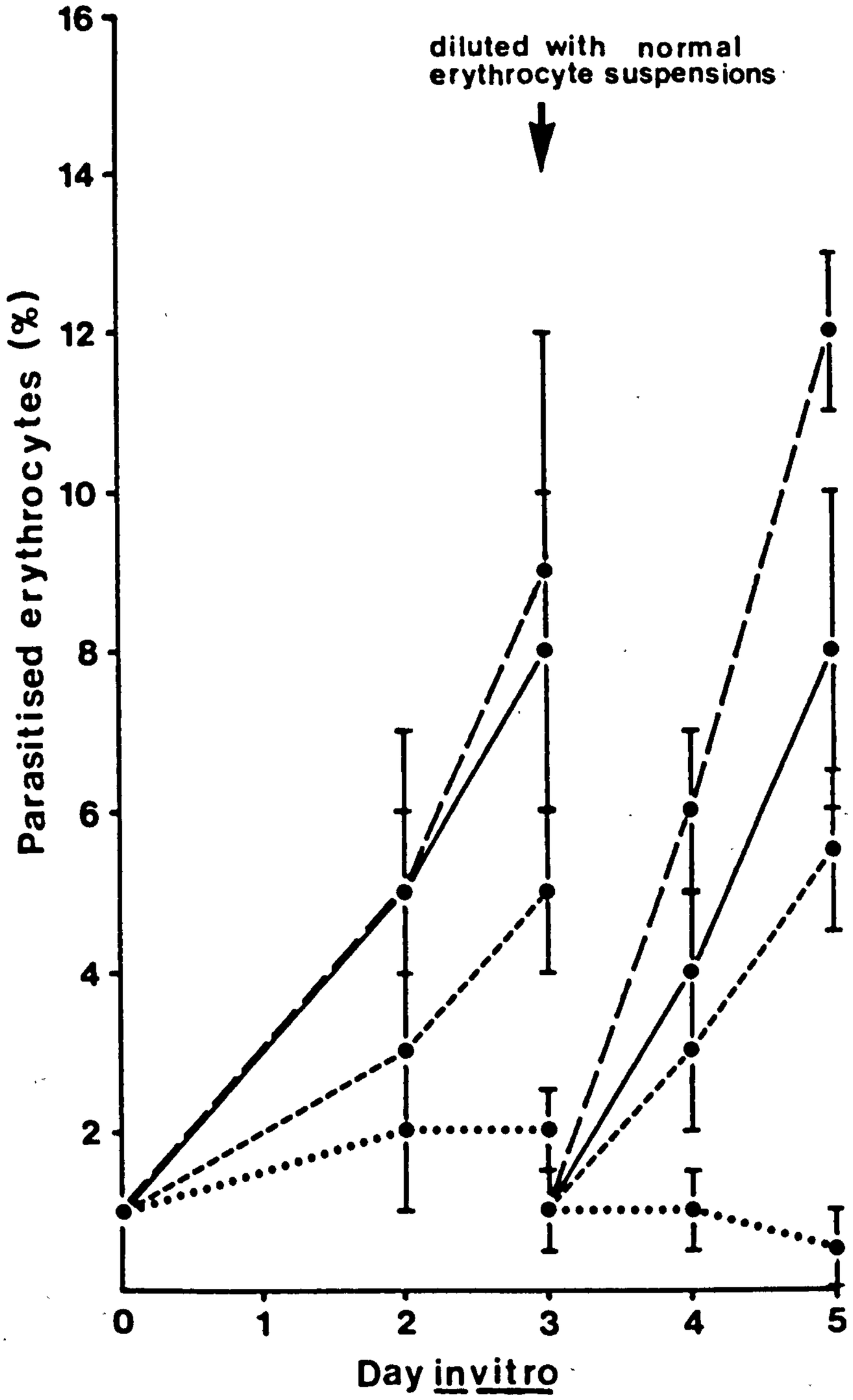
In cultures with MEM-E, RPMI 1640 or L-15 the parasitaemia remained below 5% and gradually decreased over the five day period.

There was no significant difference in the erythrocyte counts between the estimations and the counts made at random of 12 fields on Giemsa stained cytocentrifuge smears (Appendix 18).

8.3 Incorporation of Tritiated Nucleic Acid Precursors by B. bovis In Vitro

8.3.1 Introduction: Pioneer experiments on nucleic acid metabolism by Plasmodium vinckei and P. berghei (Büngener and Nielson, 1967, 1968), P. lophurae (Walsh and Sherman, 1968; Trager, 1971) and P. knowlesi (Gutteridge and Trigg, 1970) were based on the incorporation of tritiated nucleotide precursors by the parasites either immediately after extraction from infected animals or in short-term cultures. The introduction by Trager and Jensen (1976) of a method for the cultivation of P. falciparum has facilitated the application of radioisotopic methods to further elucidate mechanisms of purine biosynthesis (Ting and Sherman, 1981; Webster and Whaun, 1981) and to quantitatively assess the effect of exogenous factors on parasite growth (Desjardins, Canfield, Haynes and Chulay, 1979; Perrin, Ramirez, Lambert and Miescher, 1981; Chulay, Haynes and Diggs, 1983; Jensen, Boland, Allan, Carlin, Vande Waa, Divo and Akood, 1983).

Figure 8.2 Growth of Babesia bovis (Mexico) in different complete media.
Figures are means ± standard deviations of counts from 3 cultures.


diluted with normal erythrocyte suspensions
Parasitised erythrocytes (%)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Day invitro
M199 /40NBS
M199 / 20NBS
MEM-H / 20 NBS
MEM-E / 20 NBS

Similar techniques have been employed to evaluate the activity of drugs against Babesia by measuring the inhibition of tritiated adenosine (Chiodini, 1973b) and hypoxanthine (Irvin and Young, 1977, 1978) incorporation. The usefulness of the system was limited, however, by the apparent cessation of parasite metabolism, hence isotope uptake, after 6-12 hours in vitro (Irvin et al., 1978; Irvin and Young, 1979).

A semi-automated microdilution technique, developed for screening drugs in P. falciparum cultures (Desjardins et al., 1979) was modified for the B. bovis incorporation experiments described in this section. The 24 hour incorporation period was retained because it was convenient and potentially the most applicable to further studies on the effect of immunological factors on B. bovis and, prospectively, on Theileria. The uptake by B. bovis of tritiated purine and pyrimidine precursors in the first 24 hours after culture establishment was compared to the level of incorporation when isotopes were added after 24 hours in vitro.

8.3.2 Materials and methods: Cultures of the Mexican and South African isolates of B. bovis were established for each experiment as described in Section 8.2.2 except that the final erythrocyte suspensions were in MEM-H/20 NBS without antibiotics or additional l-glutamine. The normal bovine erythrocyte suspensions, used to dilute the B. bovis cultures to parasitaemias of 1-2% also provided control culture suspensions. The uninfected and B. bovis-infected erythrocyte suspensions were deposited in 200 µl aliquots into microtitre wells on duplicate plates. The mean number of erythrocytes per ml in the culture suspensions was 2.4×10^9, with a standard deviation of 6×10^7.

The isotope solutions were prepared and added to the cultures, as described in Section 2.13.1.

The protocol is summarised below:

I. First incorporation period

200 µl culture + 25 µl isotope solution —Incubate 24 hour→ Harvest culture

II. Second incorporation period

200 µl culture + 25 µl medium —Incubate 24 hour→ remove 175 µl medium; replace with 150 µl medium and 25 µl isotope solution —Incubate 24 hour→ Harvest culture

Each isotope solution was added to six replicate cultures of each B. bovis isolate and six uninfected erythrocyte control cultures on separate plates for periods I and II. The cultures were gassed with 5% CO_2 in air, in plastic incubation boxes and maintained at 37°C.

Evaluations were based on the examination of Giemsa stained cytocentrifuge smears and liquid scintillation counts, as described in Section 2.13.2.

8.3.3 Results: The results of preliminary experiments, in which the incorporation of tritiated hypoxanthine and adenosine was evaluated, are summarised in Appendix 19.

The results given in this section have been pooled from two separate trials. The mean values of parasite counts made from cultures with and without added isotope solutions are displayed in Table 8.2. Mean values of the pooled liquid scintillation counts are displayed in Figures 8.3 and 8.4 for the South African isolate and in

Figures 8.5 and 8.6 for the Mexican isolate. The mean scintillation counts from the two trials are tabulated in Appendix 20.

The percent infected erythrocytes increased in the cultures from 1-2% to 3-5% at 24 hours and 6-8% by the last evaluation, 48 hours after culture establishment (Table 8.2). Parasitaemia counts made from cultures with tritiated adenosine or hypoxanthine were similar to the counts from corresponding cultures without isotopes.

There was a marked increase in the uptake of all the purine nucleotide precursors as compared to the controls (Figures 8.3 and 8.5). Statistical analysis was not deemed necessary as the differences between the counts derived from cultures with B. bovis and from the control uninfected erythrocytes were obviously highly significant. The highest scinillation counts were consistently in the cultures with either hypoxanthine or adenosine. The counts made of cultures which received purine isotopes, particularly adenine and guanosine, after the first day in vitro (Period II) were higher than scintillation counts of cultures incubated during the initial 24 hours with isotopes (Period I).

The uptake of the pyrimidine bases, shown in Figures 8.4 and 8.6 as cpm x 10^2, was less than that of the purine precursors, expressed as cpm x 10^3. The difference between the control cultures and B. bovis cultures of both isolates which received uridine and cytidine was highly significant (Table 8.3). The scintillation counts from B. bovis cultures incubated in the second incorporation period with uridine and cytidine were higher than from the first period. The difference in the uptake of thymidine in B. bovis and in control cultures was not significant.

Table 8.2 Number of erythrocytes infected with Babesia bovis per 1000 counted in culture samples from Experiment 8.3

Day in vitro	Sample	
	South African strain	Mexican strain
Trial 1		
Day 0	15 ± 2	10 ± 2
1	38 ± 3	31 ± 2
2	68 ± 5	58 ± 4
Trial 2		
Day 0	20 ± 3	15 ± 3
1	48 ± 5	36 ± 2
2	79 ± 6	77 ± 5

Values are the mean ± standard deviation of counts from samples of 24 cultures.

Legend for Figures 8.3-8.6

Control uninfected erythrocyte cultures

B. bovis cultures - Period I

B. bovis cultures - Period II

All figures displayed represent means ± standard deviations of 6 replicate cultures in 2 separate trials.

Figure 8.3 Liquid scintillation counts per minute (C.P.M.) of Babesia bovis (South Africa) cultures after 24 hours incubation with tritiated purines.

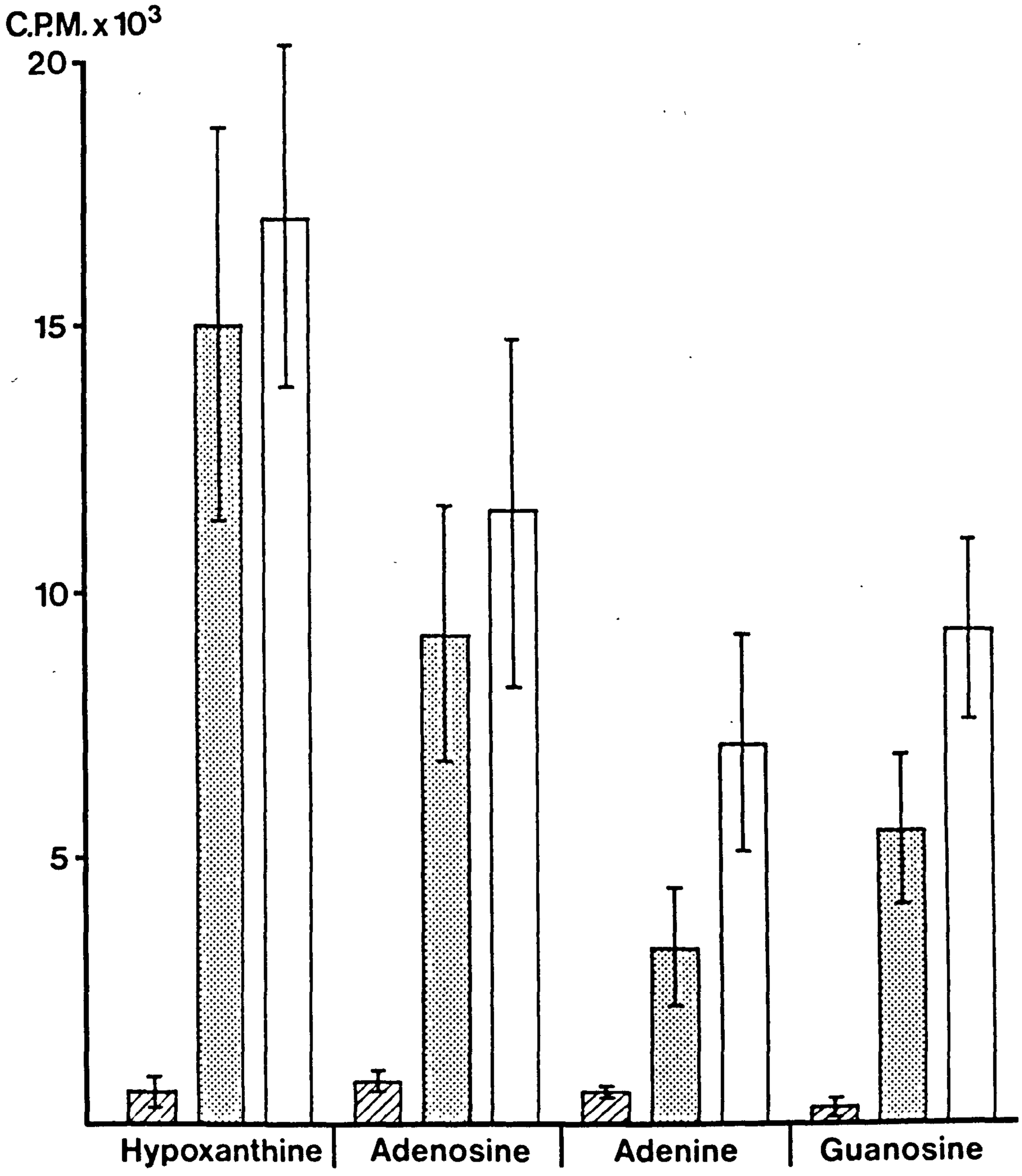

C.P.M. x 10³
20
15
10
5
Hypoxanthine
Adenosine
Adenine
Guanosine

Figure 8.4 Liquid scintillation counts per minute (C.P.M.) of Babesia bovis (South Africa) cultures after 24 hours incubation with tritiated pyrimidines.

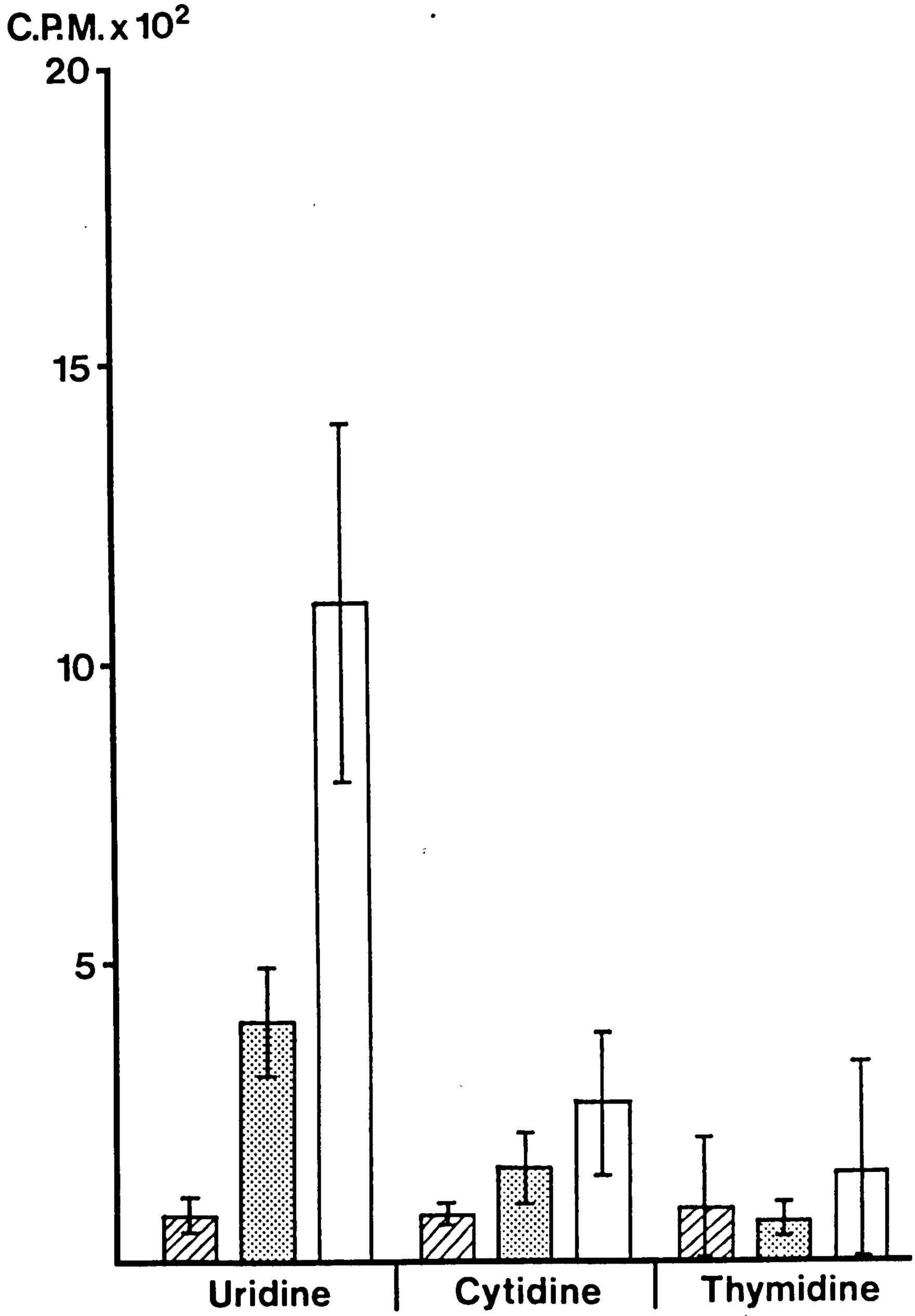

C.P.M. x 10²
20
15
10
5
1
Uridine
Cytidine
Thymidine

Figure 8.5 Liquid scintillation counts per minute (C.P.M.) of Babesia bovis (Mexico) cultures after 24 hours incubation with tritiated purines.

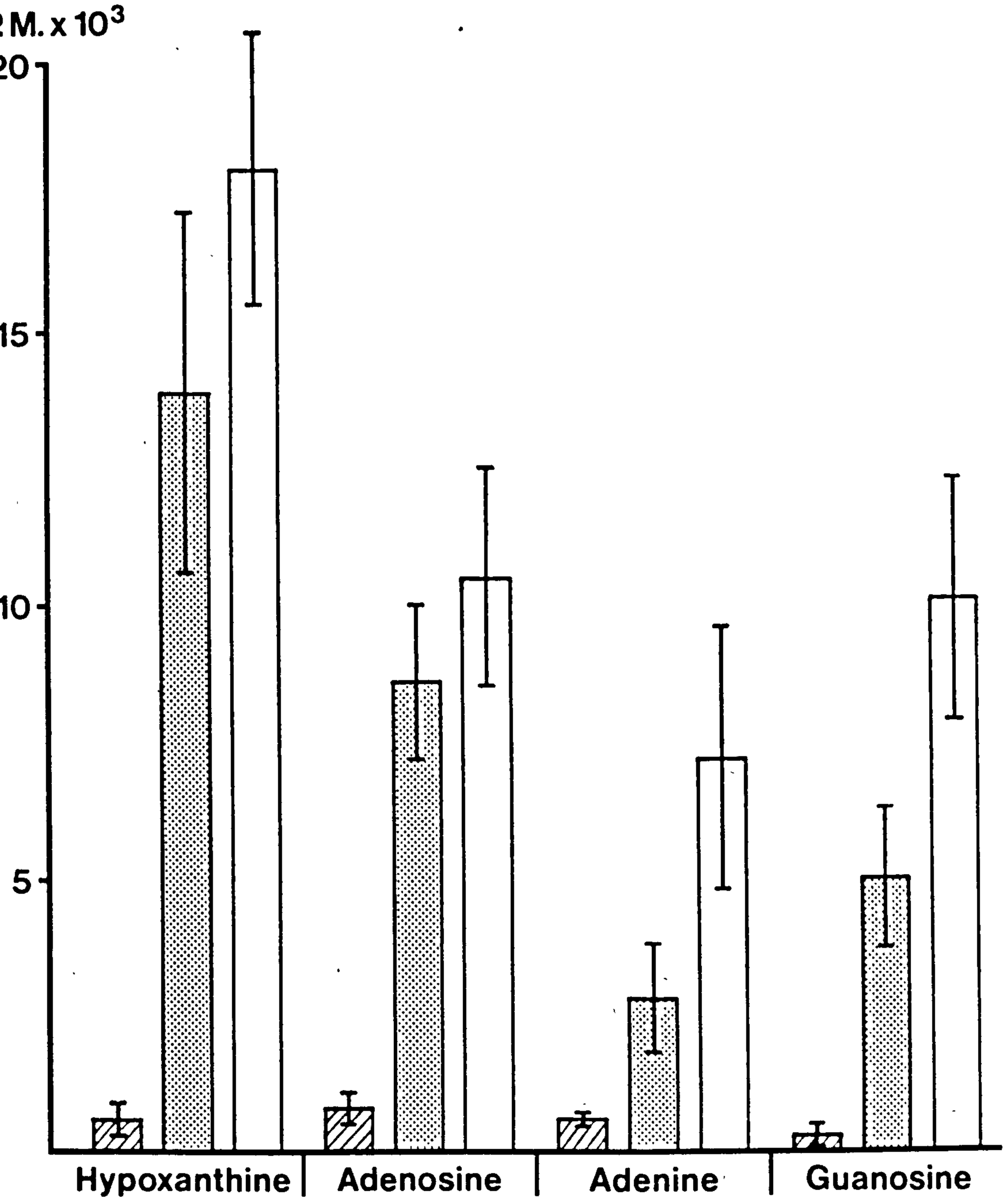

C.P.M. x 10³
20
15
10
5
Hypoxanthine
Adenosine
Adenine
Guanosine

Figure 8.6 Liquid scintillation counts per minute (C.P.M.) of Babesia bovis (Mexico) cultures after 24 hours incubation with tritiated pyrimidines.

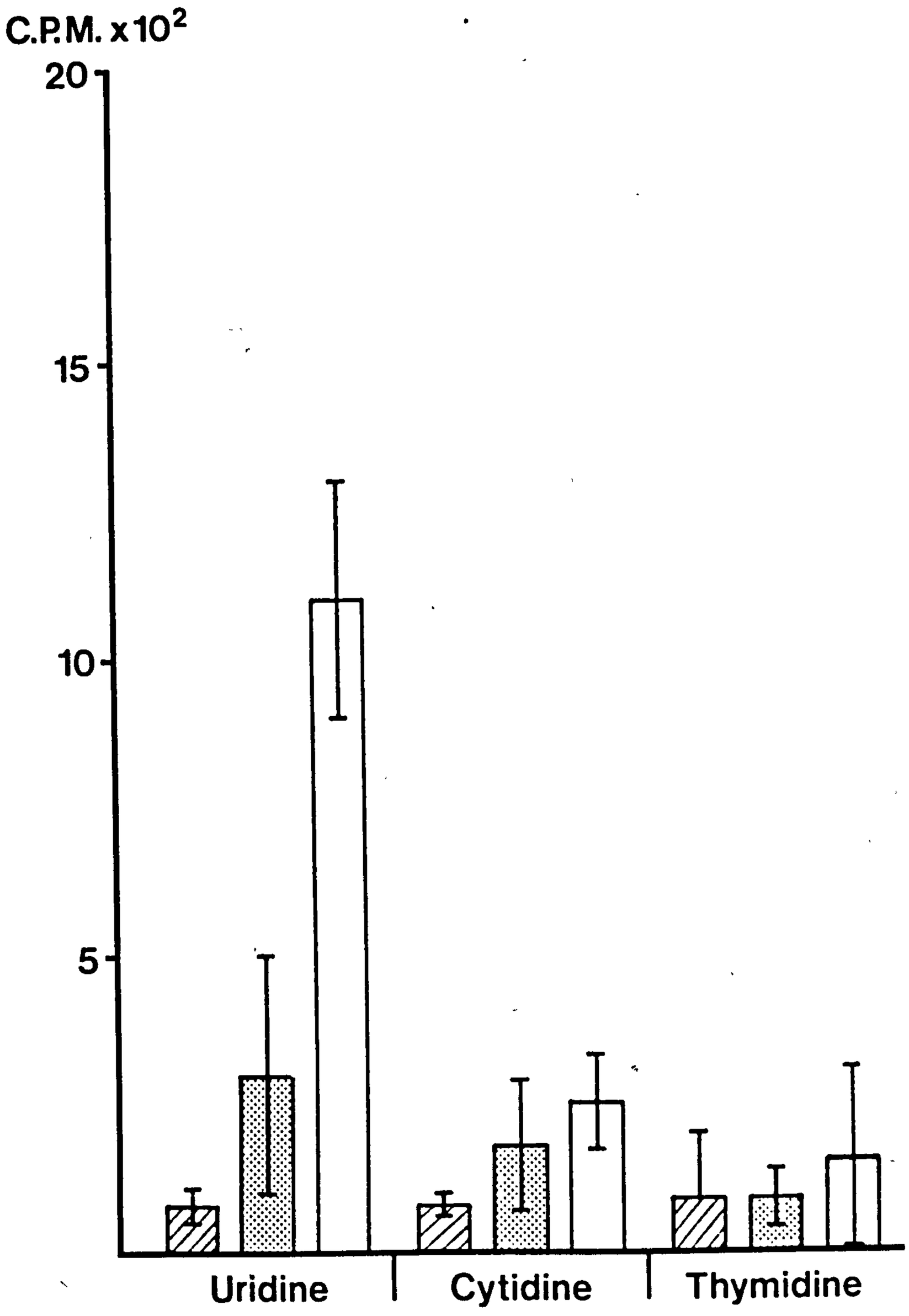

C.P.M. x 10²
20
15
10
5
Uridine
Cytidine
Thymidine

Table 8.3 Uptake of tritiated uridine and cytidine in Babesia bovis cultures compared to control cultures of uninfected bovine erythrocytes. Student's t-test values* (df = 22)

Babesia bovis cultures	Uridine	Cytidine
Mexico		
Period I	5.0	3.3
Period II	18.6	6.2
South Africa		
Period I	11.6	4.7
Period II	10.4	5.1

All of the t values displayed are highly significant ($P < 0.01$)

*Based on the mean liquid scintillation counts from two trials, each with six replicate cultures per comparison.

8.4 Discussion

B. bovis was isolated from the blood of infected calves with parasitaemias ranging from less than 0.1% to 15% (Section 8.1), maintained continuously for 3 months in stationary erythrocyte cultures and utilized for studies on the incorporation of tritiated nucleic acid precursors. Isolations from low parasitaemia blood appeared to benefit from the presence of pre-established BAE monolayers which suggested that this technique may be useful in overcoming the problems of a minimum seeding density for B. bovis in MASP cultures (Levy and Ristic, 1980).

Higher peak parasitaemias were consistently obtained in B. bovis cultures with M199 and either 20 or 40% NBS than in any of the other complete media tested in a series of comparative experiments in Section 8.2. Both isolates of B. bovis were maintained for several (3) passages in MEM-H/20 NBS, which was the complete medium selected for incorporation experiments. Unlike M199, preformed purine and pyrimidine bases were not included in MEM. Observations made by the author during preliminary experiments confirmed that the incorporation of hypoxanthine was at least 1.7 times greater in B. bovis cultures with MEM-H/20 NBS than with M199/20 NBS.

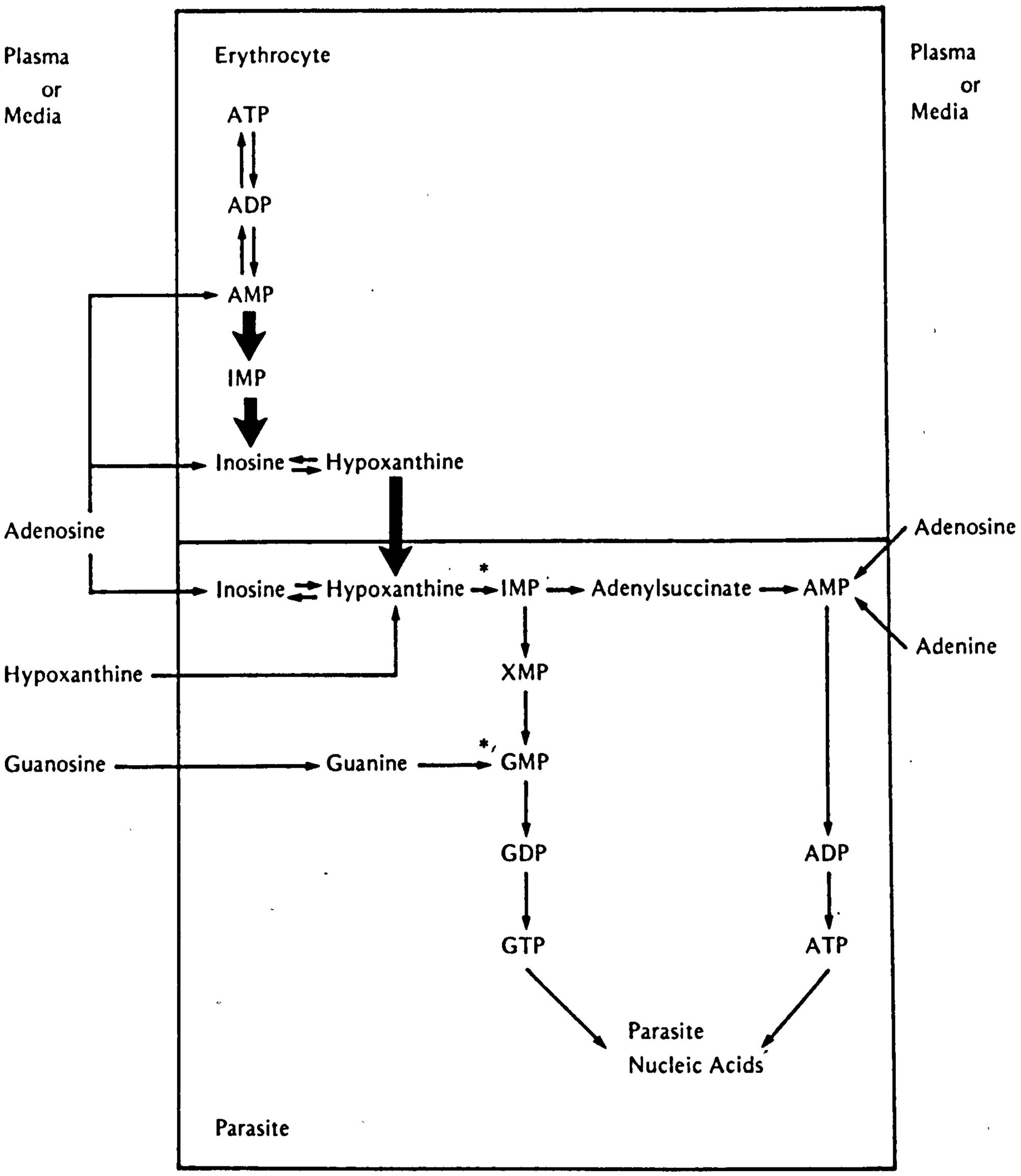
The experiments described in Section 8.3 indicated that tritiated hypoxanthine, adenosine, adenine, guanosine, uridine and cytidine were incorporated by the two B. bovis isolates in vitro (Figures 8.3-8.6). The uptake of purine nucleotide precursors, particularly hypoxanthine and adenosine, was markedly greater than that of the pyrimidines. Thymidine did not appeared to be utilized by the parasites, as counts from B. bovis cultures were never significantly different from the controls.

The uptake of purines by B. bovis was not unexpected as the ability of organisms to incorporate exogenous purines is almost ubiquitous (Hitchings, 1982). The incorporation of radiolabelled hypoxanthine and adenosine by species of Plasmodium (Van Dyke, 1975; Hansen, Sleeman and Pappas, 1980) and Babesia (Irvin et al., 1978; Irvin and Young, 1979) was consistently greater than that of adenine or guanosine.

The synthesis of purine nucleotides by intraerythrocytic protozoa has been studied most extensively in Plasmodium. The principle purine metabolic pathway for intraerythrocytic malarial parasites involves the conversion of hypoxanthine to inosine monophosphate (IMP) in a reaction catalysed by hypoxanthine-guanine phosphoribosyltransferase as reviewed by Königk (1977), Sherman (1979) and Homewood and Neame (1980). IMP is then converted through a series of reactions to the nucleic acid precursors, adenosine triphosphate (ATP) and guanosine triphosphate (GTP), as illustrated in Figure 8.7. The GTP precursor, guanosine 5' monophosphate (GMP) can also be synthesised from guanosine, via guanine, in a reaction catalysed by hypoxanthine-guanine phosphoribosyltransferase (Tracy and Sherman, 1970; Yamada and Sherman, 1981a; Reyes, Rathod, Sanchez, Mrema, Riekmann and Heidrich, 1982).

The utilization by B. bovis of preformed purines, particularly hypoxanthine, via pathways analogous to those used by Plasmodium seems logical when considering the availability of these substrates. Hypoxanthine and adenosine are the major purines produced by nucleotide degradation in the liver, transported by erythrocytes and salvaged for nucleic acid synthesis by mammalian cells (Murray, 1971;

Figure 8.7 Purine salvage pathways proposed for intraerythrocytic stages of Plasmodium.
Diagram based on Sherman (1979), Ting and Sherman (1981), Yamada and Sherman (1981a,b) and Reyes et al. (1982).



*Hypoxanthine-guanine phosphoribosyltransferase

Fox and Kelly, 1978). Hypoxanthine, utilized for parasite purine biosynthesis, is derived by facilitated diffusion from the extracellular environment (Ting and Sherman, 1981), catabolism of ATP in the host erythrocyte (Beutler, 1974; Grimes, 1980) or conversion of adenosine (Van Dyke et al., 1977; Hansen et al., 1980; Webster and Whaun, 1981). The formation of hypoxanthine via inosine in the erythrocyte is favoured either at high adenosine concentrations or as ATP catabolism is accelerated to satisfy the erythrocyte's metabolic requirements (Grimes, 1980; Hawkins, Kyd and Bagnara, 1980; Yamada and Sherman, 1981b). There seems, therefore to be an obvious advantage gained by any intraerythrocytic parasite that can salvage a readily available substrate, such as hypoxanthine, which is a degradative end product of host cell metabolism.

The increase in the uptake of hypoxanthine and adenosine during the second incorporation period was not as great as that of adenine or guanosine. The indication from these results, and ancillary experiments with shorter incorporation periods (Appendix 21) was that at the higher parasitaemias in Period II most of the available hypoxanthine and adenosine was incorporated in less than 24 hours.

Results of the experiments with B. bovis suggest that, in vitro, purine nucleotide biosynthesis occurs via salvage pathways. Several attempts to produce autoradiographs in conjunction with these experiments in order to localize the site of incorporation within the parasite were unsuccessful. The preformed purines are probably metabolised for RNA and DNA production, but further studies are required to confirm this supposition.

The uptake of uridine and cytidine by B. bovis was observed in cultures of the two isolates in both incorporation periods and in repeated trials. The uptake of pyrimidines by autologous uninfected erythrocytes and leucocytes in the control cultures was minimal. If stimulated lymphocytes in the B. bovis cultures were incorporating uridine and cytidine, one would have expected a concurrent increase in thymidine uptake (Peters and Veerkamp, 1983).

Most parasitic protozoa are capable of synthesising pyrimidines de novo and by salvage pathways (Gutteridge and Coombs, 1977). De novo biosynthesis of pyrimidines from glutamine, adenosine triphosphate and bicarbonate ions, to uridylic acid (UMP) with the subsequent formation of cytidylic (CMP) and deoxythymidylic (dTMP) acids is propounded to be the predominant pyrimidine pathway utilized by intraerythrocytic malarial parasites (Sherman, 1979; Hill, Kilsby, McIntosh, Wrigglesworth and Ginger, 1981; Reyes et al., 1982). P. knowlesi, P. berghei and P. falciparum have, however, been shown to incorporate tritiated uridine in vitro (Conklin, Chou, Siddiqui, and Schnell, 1973; Grothaus, Castilla, Müller and Kreier, 1982). B. rodhaini incorporated uridine and thymidine more readily than orotic acid (Irvin et al., 1978; Irvin and Young, 1979). However, identification of five of the six enzymes of the pyrimidine de novo biosynthetic pathways indicates that B. rodhaini is not dependent on pyrimidine salvage (Holland, Gero and O'Sullivan, 1983).

The uptake in B. bovis cultures of uridine and cytidine at relatively low levels, as compared to the purines, suggests that the salvage pathways were employed when metabolic requirements were not satisfied by de novo synthesis. The exhaustion of the precursor

nutrients, such as glutamine, may account for the necessity to utilize preformed pyrimidine bases *in vitro*.

The uptake of radiolabelled pyrimidines and purines *in vitro* implies, but does not prove, that the metabolites are being utilized by *B. bovis* for nucleic acid synthesis. Some of the radioactivity measured in the cultures may be attributable to the localisation of isotopes in parasitized erythrocytes which may be more resistant to osmotic lysis in the cell harvesting procedure (Mahoney, 1967). Biochemical studies comparing the uptake of isotopes by extracellular and intraerythrocytic *B. bovis* merozoites, as well as enzyme analysis would be useful adjuncts to this investigation.

The *B. bovis* cultures were originally established so as to provide a readily available source of parasites which could be used to develop techniques required for studies on theilerial piroplasms.

The results of the isotope incorporation studies described in this chapter suggest that the methodology employed may have practical applications in further studies on *B. bovis*. The inhibition of hypoxanthine or adenosine uptake by *B. bovis in vitro* could potentially be used to measure the effect on the parasite of exogenous factors, such as drugs, monoclonal antibodies or soluble immune factors. The simple invasion assay techniques used in Chapter 4, in conjunction with isotope incorporation studies might help to distinguish the specific effect of immune factors that inhibit merozoite invasion and/or the intraerythrocytic development of *B. bovis*.

CHAPTER NINE

GENERAL DISCUSSION

At the outset of this project the mode of intraerythrocytic multiplication of Theileria annulata was not clear, and there were doubts whether the piroplasms of T. parva could divide. Isolation of the parasites in stationary erythrocyte cultures made it possible to show, by light and electron microscopy, that both T. annulata and T. parva multiplied within the erythrocytes by a process of schizogony.

The small parasites in quadruplet forms seen in this study had the component structures of the apical complex of infective merozoites (Aikawa and Sterling, 1974; Scholtyseck, 1979), and yet they did not invade other erythrocytes in vitro. In the case of T. annulata the merozoites seemed unable to leave the erythrocytes after division. This raised the question as to whether division into quadruplet forms was the true mode of multiplication for T. annulata, or merely an artefact of in vitro cultivation.

While there is no doubt that quadruplet forms occur in the blood of cattle infected with T. annulata, their significance has not been clearly demonstrated. The invasion of erythrocytes by merozoites that are produced by microschizonts during the course of infection results in a progressive increase in the number of multi-parasitized erythrocytes. Erythrocytes with quadruplet forms constitute only a small proportion, generally less than 1%, of the parasitized erythrocytes in the blood of infected cattle.

The protocol used by Neitz (1959, 1964) to study the multiplication of T. mutans and T. parva piroplasms, by the inoculation of

blood from chronic carriers into splenectomized cattle, served as the pattern for an experiment designed to determine the primary mode of intraerythrocytic multiplication of T. annulata in vivo. Quadruplet forms, identical to those observed in vitro, were present in the blood of splenectomized carriers and of splenectomized calves which were inoculated with T. annulata-infected blood from one of the carrier calves. The relative proportion of erythrocytes with quadruplet forms increased progressively in all four calves during the initial parasitaemic peak.

Analysis of parasite counts in fresh blood smears showed that at parasitaemias of 15-20%, the probability that four parasites in an erythrocyte were due to multiple merozoite invasion was extremely low. In these calves the number of erythrocytes with two parasites was consistently higher than the number with four. Nevertheless, the incidence of paired piroplasms in erythrocytes was never significantly greater than the number which could be attributed to the random invasion of merozoites produced from microschizonts.

The results of in vitro and in vivo studies thus indicated that the primary mode of intraerythrocytic multiplication for T. annulata was by schizogonous division into four merozoites. The parasite strains examined were originally isolated in Turkey and India. T. annulata isolated in Israel has been seen to multiply by the same process in stationary erythrocyte cultures established using blood at parasitaemia levels of 3.5 and 7.0% (E. Pipano, personal communication). In that study a three to four-fold increase in the parasitaemias was reported to occur in vitro but the cultures were not maintained continuously beyond day 17-20 post establishment.

In depth *in vivo* studies on *T. parva* were not feasible within the time span of this investigation. However, the indication from *in vitro* cultivation experiments, electron microscopic studies and the examination of blood smears from acutely infected calves was that the Muguga strain of *T. parva* divided within the erythrocytes by the same schizogonous process as *T. annulata*. Essentially, these observations corroborate the mode of multiplication proposed by Koch (1905) and Dschunkowsky (1927) who first described the formation of four anaplasmoid *T. parva* and *T. annulata* parasites, respectively, in the erythrocytes of infected cattle.

There is evidence to suggest that schizogonous division into four intraerythrocytic merozoites is, as Bettencourt *et al.* (1907) astutely noted, a characteristic of *Theileria*. Thus far, similar quadruplet forms have been reported in an impala with theileriosis (Grootenhuis, Young, Kimber and Drevemo, 1975), in eland infected with *T. taurotragi* (Grootenhuis, 1979) and in cattle infected with *T. mutans* (Wenyon, 1926, 1965, see Figure 1.4; Oteng, 1971), *T. annulata* (Dschunkowsky, 1927; Sergent *et al.*, 1945; Srivastava and Sharma, 1976a), *T. parva lawrencei* (de Vos, 1982), *T. parva parva* (Koch, 1905; Nuttall *et al.*, 1909, see Figure 1.3; Cowdry and Ham, 1932; Cowdry and Danks, 1933) and a *Theileria* species in Japan (Ishihara and Ishi, 1958). The latter is probably a member of the *T. sergenti/orientalis* group (Uilenberg, 1981).

The same mode of multiplication was described by Nuttall and Strickland (1912) for *Nuttallia equi*, later reclassified as *Babesia equi* (Neitz and Jansen, 1956), as shown in Figure 1.2. Division into four merozoites has since been confirmed as the primary mode of

multiplication for B. equi, using phase-contrast microscopy, time lapse cinematography, and electron microscopy (Simpson, Bild and Stoliker, 1963; Holbrook, Johnson and Madden, 1968). Interestingly, intralymphocytic schizonts, like those seen in Theileria, have been identified as developmental stages of B. equi (Schein et al., 1981; Moltmann et al., 1983a) and of another haemoprotozoan, Nuttallia danii in gerbils (Meriones tristrami shawii) which also divides into quadruplet forms within the erythrocytes (Tsur, Hadani and Pipano, 1960).

In the light of several recent discoveries, the taxonomy of species within the Piroplasmida will have to be reconsidered. The characteristic ability of some piroplasms to divide into distinct intraerythrocytic quadruplet forms may be a valuable clue, indicating that a particular species is more closely related to Theileria than was previously recognised.

Babesia microti is one notable example. The process of intra-erythrocytic division into four merozoites in B. microti, revealed by electron microscopy (Rudzinska and Trager, 1977), is remarkably similar to the division of T. annulata and T. parva observed in this study. A comparison between the observed distribution of B. microti in blood samples prepared from an infected mouse and Poisson distribution showed a correlation similar to comparisons made using counts of T. annulata in erythrocytes of the recipient calves 163 and 164 (analysis of B. microti data provided by A.D. Irvin). B. microti, like B. equi, N. danii and Theileria spp., is transmitted trans-stadially and not transovarially by ixodid ticks (Friedhoff and Smith, 1981; Hadani, 1981; Piesmann and Spielman, 1982). In addition,

sporogony in the salivary glands of the tick vector and the fine structure of the sporozoites of B. microti (Karakashian, Rudzinska, Spielman, Lewengrub, Piesman and Shoukrey, 1983) have been shown to resemble T. parva (Fawcett et al., 1982) and B. equi (Moltmann, Mehlhorn, Schein, Voigt and Friedhoff, 1983b) more closely than they resemble other babesial parasites.

Similarities to Theileria and to B. equi point to the possibility that B. microti may also have an intralymphocytic stage of development. The discovery in the life cycle of B. microti of an exoerythrocytic schizont stage, capable of inducing blast-transformation and abnormal proliferation of the host lymphoid cell, would be particularly significant since the parasite is known to infect man (Western, Benson, Gleason, Healy and Schultz, 1970; Spielman, 1976; Spielman and Piesman, 1979; Dammin, Spielman, Benach and Piesman, 1981). Certainly, detailed investigations with special consideration being given to the small babesial parasites that form "maltese cross" or quadruplet forms, such as B. microti and B. felis, are required before a major taxonomic revision of the Piroplasmida can be advocated.

The relationship between theilerial and babesial parasites may also be of epidemiological significance. The concept of strict host specificity for many of the piroplasm parasites is no longer dogmatically accepted and questions have been raised as to whether wild animals might act as unrecognised reservoir hosts for these piroplasms (Arthur, 1966; Barnett and Brocklesby, 1968; Purnell, 1981). Arthur (1966) went so far as to suggest that B. microti and B. equi may be the same parasite. He reasoned that ticks feeding as larvae or nymphs on infected rodents or insectivores could, after moulting, infect

other rodents or large animals, such as horses. Four of the seven vectors of B. equi feed in their immature stages on rodents (Hoogstraal, 1956; Arthur, 1966). One of these ticks, Hyalomma excavatum, can also transmit T. annulata (Samish and Pipano, 1983) and N. danii (Tsur et al., 1960). These facts suggest that consideration should be given to the possibility that piroplasms exhibiting characteristic quarternary division in the erythrocytes of different hosts could, in some cases, be the same parasite species.

Useful information for epidemiological studies might be obtained if the relationship between piroplasms isolated from different geographical areas were determined, using more specific criteria than the morphological appearance of parasites in light microscopic examinations and their antigenicity in conventional serological tests. If a continuous cultivation system were devised for Theileria, similar methodology might be successfully applied to grow field isolates of piroplasms which multiply by the same process, for further comparative analysis and identification.

Isolations could be made, for example, from white-footed mice (Peromyscus leucopus), white-tailed deer (Odocoileus virginianus) and human inhabitants of Nantucket Island, where a high incidence of human babesiosis, caused by B. microti, has been reported (Spielman and Piesman, 1979). If a sufficient number of parasites were made available by in vitro cultivation, their true relationship might be determined by isoenzyme (Carter, 1978; Momen, 1979; Momen, Chance and Peters, 1979; Melrose and Brown, 1979; Allsopp and Gibson, 1983) or genetic analysis techniques (Borst, Fase-Fowler and Gibson, 1981; Frasch, Goijman, Cazzulo and Stoppani, 1981; Goman, Langsley, Hyde, Yankovsky, Zolg and Scaife, 1982).

The original aim of this study, to establish a continuous cultivation system for the intraerythrocytic stages of Theileria, was not achieved. The need to infect cattle to obtain parasitized erythrocytes limited the number of cultivation experiments which could be conducted with T. annulata and T. parva in the time available. However, cultures of a B. bovis strain from Mexico, which was known to be capable of continuous growth in vitro (Erp et al., 1978; Levy and Ristic, 1980), and of a new South African isolate were established. The B. bovis cultures provided experience in handling continuous cultures and an abundant source of intraerythrocytic parasites for experiments.

The B. bovis culture system was used to develop techniques for invasion assays, electron microscopy and radioisotope incorporation which could then be applied to Theileria. The last mentioned technique was used as a means of assessing the nucleic acid metabolism and growth of T. annulata in vitro. Results of the T. annulata experiments are not included in this thesis because inexplicably high scintillation counts from control cultures cast doubt on their validity. Similar problems were not encountered in the incorporation experiments with B. bovis and these results were readily reproducible.

Continuous cultures provided a plentiful supply of two B. bovis strains which were used to expand the study on nucleotide precursor incorporation, to prepare antigens for serodiagnostic tests and to investigate the presence of parasite specific isoenzymes (Appendices 22-26). Experience with B. bovis clearly demonstrated the multitude of potential applications of in vitro cultivation systems for intraerythrocytic protozoa. The paucity of information on the

basic biochemical structure and metabolism of babesial and theilerial piroplasms emphasises the need for further biochemical studies, which would be markedly facilitated by methods for the long-term cultivation of these parasites.

Exciting areas of current investigation are focussing on the determination of factors, including specific immunoglobulins, monokines and other soluble effector factors, which inhibit the infectivity and/or growth of intraerythrocytic merozoites of some species of Babesia (Clark, 1976; Bautista and Kreier, 1980) and Plasmodium (Taverne et al., 1982; Clark and Hunt, 1983; Haidaris, Haynes, Meltzer and Allison, 1983; Jensen et al., 1983). In vitro culture systems could be used to test the effect of soluble immune factors or monoclonal antibodies on the growth of bovine Babesia and Theileria before incurring the expense of cattle experiments. The invasion assay and radioisotope incorporation techniques might prove to be useful adjuncts to the fundamental parasite counts in determining the specific effect of these humoral and cell mediated immune factors, as well as chemotherapeutic agents.

During the course of this study several stimulating questions have been raised, relevant to Theileria, which provide impetus for a continued effort to establish an in vitro cultivation system for piroplasms. The most immediate questions are those pertaining to the role of the intraerythrocytic merozoites of T. annulata and T. parva. Are these merozoites destined to invade other erythrocytes, develop as trophozoites and subsequently divide by schizogony to form more infective merozoites? Could some, or perhaps all, of these merozoites be capable of invading other cells, including lymphocytes,

where they would undergo schizogony to form merozoites which would then infect erythrocytes? The first step towards answering these questions could be to determine the susceptibility of different cell types to merozoite invasion.

The problems of controlling cattle movement and the potential for carrier cattle, in the presence of the appropriate tick vectors, to disseminate theileriosis emphasises the importance of investigating the nature of the carrier state. Present evidence, reviewed by Pipano (1981) and Allison (1981), suggests that a protective immune response to homologous challenge with Theileria is dependent upon the establishment of macroschizonts in the lymphoid cells of vaccinated animals. Current vaccination protocols are based either on the inoculation of culture-derived infected lymphoid cells (Brown, 1981) or infection with sporozoites followed by drug treatment (Radley, 1981). The potential importance of chronic T. parva carriers resulting from vaccination or after clinical treatment has recently become apparent (Young et al., 1981; Dolan, 1981). A more complete knowledge of the host-parasite relationship would help in determining the role of intraerythrocytic schizogony in maintaining the carrier state and contributing to clinical relapses after vaccination or chemotherapy.

Another basic parasitological question, potentially important in the control of theileriosis, is the relationship between the intraerythrocytic parasites described in this thesis and the supposed sexual stages of Theileria. Koch (1905, 1906) originally proposed that the "Strahlenkörper" he observed in ticks infected with B. bigemina and T. parva were sexual stages of these parasites. Nonetheless, the existence of sexual stages in the development of Babesia

and Theileria remained controversial for nearly 70 years (Neitz, 1965; Levine, 1971). In the past decade "Strahlenkörper" have been identified in ticks infected with T. annulata (Schein, 1975; Schein et al., 1975; Mehlhorn et al., 1975), T. taurotragi (Young, Grootenhuis, Leitch and Schein, 1980), T. mutans (Warnecke, Schein, Voigt, Uilenberg and Young, 1980), T. velifera (Warnecke, Schein, Voigt and Uilenberg, 1979), B. bovis (Stewart, 1978) and B. microti (Rudzinska, Spielman, Riek, Lewengrub and Piesman, 1979), as well as T. parva (Mehlhorn and Schein, 1976; Schein et al., 1977) and B. bigemina (Friedhoff and Büscher, 1976; Dalgliesh, Stewart and Rodwell, 1981). The hypothesis that the diminutive "Strahlenkörper", with spiky processes, are male microgametes and the rotund forms are female macrogametes appears to have gradually gained acceptance.

In conjunction with T. annulata and T. parva cultivation experiments the parasites' development in tick tissue cultures was also studied. The transmission experiments in Chapter 6 showed that the inoculation of piroplasm culture suspensions into cattle would not be a valid means of assessing the viability of cultivated parasites unless all non-erythrocytic cells could be removed without having a deleterious effect on the piroplasms in the inocula. In an attempt to determine whether parasites could develop to gametocytes after being maintained in vitro for six days, culture suspensions were transferred to cultures with and without nymphal tick gut preparations (Bell, 1983) and incubated at 28°C. The development of T. annulata and T. parva, previously cultivated at 37°C, was studied for a period of six days in the 28°C cultures, and compared to parasite development in control cultures at 28°C which had been established with blood

taken directly from infected cattle.

Parasitic forms identical to those described as microgametes and macrogametes of Theileria were observed in both 28°C cultures established with T. annulata and T. parva, after having been cultivated at 37°C for six days,and in the control cultures (P.A. Conrad and L.J. Bell, unpublished observations). Distinct spiky projections were seen on individual intraerythrocytic and extracellular parasites, but were never seen on parasites in quadruplet forms. The parasite population which developed into spiky microgamete forms and the merozoites in quadruplet forms appeared to be discrete.

The parasites in 28°C cultures were morphologically similar to forms seen in Giemsa stained tick gut smears, prepared as controls, using nymphs which engorged on the infected calves who donated the blood originally used to establish cultures at both temperatures (Bell, 1983). The tick culture experiments showed that after maintenance in stationary erythrocyte cultures for six days, T. annulata and T. parva were capable of continuing their development in vitro into forms like those seen in H. a. anatolicum and R. appendiculatus ticks.

At the same time these experiments highlighted deficiencies in our understanding of sexual development in Theileria and the danger of accepting, too readily, the attractive "Strahlenkörper" hypothesis without further critical evaluation. The argument for the existence of sex in Theileria is based on the presence of dimorphic parasite forms in the tick gut, of which the fine structure of only the microgamonts and microgametes have been described (Mehlhorn et al., 1975; Mehlhorn and Schein, 1976). Microgametes appear to form extracellular

clusters, but proof of isogamy or syngamy has not been provided.

Rudzinska et al. (1979) described the ultrastructure of similar forms of B. microti with arrowhead organelles which they tentatively called gametocytes and gametes. Recent studies have shown that the forms of B. microti which acquire these characteristic arrowhead structures while in the gut of the vector Ixodes dammini, penetrate the peritrophic membrane and enter the gut epithelium of the tick without evidence of isogamy or syngamy (Rudzinska, Lewengrub, Spielman and Piesman, 1983).

If "Strahlenkörper" are sexual stages one might expect these forms to be more infective for ticks than asexual parasite stages (Piesman and Spielman, 1982). Increased numbers of "Strahlenkörper" have, however, been seen in association with a reduction in the infectivity of vaccine strains of B. bovis and B. bigemina for Boophilus microplus ticks (Stewart, 1978; Dalgliesh et al., 1981).

The lack of more substantial evidence for the existence of a true sexual process in the developmental cycle of Theileria, other than the presence of parasite dimorphism in the tick gut, must give cause for scepticism. The question of sexuality has, however, practical implications potentially important to theileriosis control. Selection, genetic recombination and cloning of T. parva strains with the requisite antigenic characters, but reduced virulence, for use in live vaccines would be conceivable if the concept of sexuality is valid (Irvin and Boarer, 1980). Consideration might also be given to identification, using monoclonal antibodies, of antigens shared by the four primary extracellular stages of Theileria; sporozoites, exoerythrocytic merozoites from microschizonts, merozoites from

intraerythrocytic schizonts and sexual stages. There may be an advantage in a vaccine which stimulates immunity to sporozoites and schizonts, thus reducing the severity of an acute infection, as well as to merozoites and gametocytes to prevent disease transmission by controlling the infectivity of carriers for ticks.

Drug resistance has not been a major problem in theileriosis because, until recently, tetracyclines were the only drugs proven to be effective against intralymphocytic schizonts. Protocols for administering new drugs, notably parvaquone and halofuginone (Dolan, 1981; Morgan and McHardy, 1982) should, however, consider that the probability of resistant parasite strains emerging and spreading between cattle populations is increased if genetic recombination can occur (Walliker, 1982; Tait, 1983). The same principle applies to the dissemination of parasite strains which could result naturally from recombination or mutation.

The experimental proof that sexual stages do occur might be obtainable using genetic analysis techniques like those applied to studies on malarial parasites, reviewed by Walliker (1982). If the intraerythrocytic stages of distinct theilerial strains were cloned *in vitro*, as recently reported for *B. bovis* (Rodriquez, Buening, Green and Carson, 1983), combinations of clones could be injected percutaneously into ticks (Schreuder and Uilenberg, 1976; Walker *et al.*, 1979). Sporozoites acquired in the next instar from infected ticks could be used to infect normal bovine lymphocytes *in vitro* and establish parasitized cell lines, which could also be cloned (Brown, 1979). Specific parasite markers such as isoenzymes

(Melrose et al., 1980; Musisi et al., 1981; Allsopp and Gibson, 1983), or possibly, specific parasite induced antigens on the lymphoid cell, detected by monoclonal antibodies (Pinder and Hewett, 1980), could be used to determine if genetic recombination had occurred.

The prospect of completing the life cycle of Theileria in vitro provided the original motivation for this research project. There is still a great deal of progress to be made if that objective is to be achieved. The development of a cultivation system capable of sustaining the infectivity of merozoites formed by schizogony either in lymphoid cells (microschizonts) or in erythrocytes (quadruplet forms) will be the key that opens the way to a host of fundamental and fascinating studies on Theileria.

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Abbreviations used for references are based on:

Williams, P.C. (1968)
Abbreviated Titles of Biological Journals,
The Biological Council,
LONDON.

and

Brown, P. and Stratton, G.B. (1964)
World List of Scientific Periodicals,
Published in the years 1900-1960,
4th Edition,
Butterworths,
LONDON.

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Appendix 1 Suppliers of Materials and Equipment

Agar Aids, 66A Cambridge Road, Stansted, Essex, England	Araldite, sodium cacodylate
Amersham International plc, White Lion Road, Amersham, Buckinghamshire, England	Radiolabelled compounds
BDH Chemicals Ltd., Poole, England	Chemical reagents
Becton-Dickinson U.K. Ltd., York House, Empire Way, Wembley, Middlesex, England	Disposable syringes, needles, petri dishes vacutainer tubes
Boots Chemical Co. plc, 1 Thane Road West, Nottingham, England	Ethium bromide
British Oxygen Company Ltd., Seafield Road, Edinburgh, Scotland	Gas mixtures
Chance Propper Ltd., Spon Lane, Smethwick, Warley, England	Glass microscope slides and coverslips
Costar, 205 Broadway, Cambridge, Massachusetts, U.S.A.	24-well Tissue Culture Cluster^{24} plates
Coulter Electronics Ltd., Harpenden, Herts., England	ZB1 Electronic particle counter and supplies
Difco Laboratories, Detroit, Michigan, U.S.A.	Glycerol for fluorescence microscopy
W. Edwards and Co., Division of British Oxygen Co. Ltd., Manor Royal, Crawley, Sussex	Speedivac pressure pump
EM Scope Laboratories, 374 Wandsworth Road, London, England	Uranylacetate, EM processing wheel
Ernst Leitz Wetzlar, GMBH, Postfach 2020, D-6, 330 Wetzler, West Germany	Compound light (Ortholux and Dialux 2) and fluorescence (Orthoplan) microscopes
Evans Medical Ltd., Greenford, Middlsex, England	Streptomycin sulphate
Gibco Europe Ltd., Trident House, P.O. Box 35, Renfrew Road, Paisley, Scotland	Complex tissue culture media, medium supplements, FBS, Nunc plastic tissue culture flasks and microtest plates
Glaxo Laboratories Ltd., Greenford, Middlesex, England	Sodium benzylpenicillin (Crystapen), Saffan

Imperial Chemical Industries Ltd., Alderley House, Alderley Park, Macclesfield, Cheshire, England	Fluothane
Jencons (Scientific) Ltd., Cherrycourt Way Industrial Estate, Stanbridge Road, Leighton Buzzard, Beds., England	Griffiths tubes
Johnson Matthey, 74 Hatton Garden, London England	Osmium tetroxide
Kodak Ltd., Distribution Northern Region, P.O. Box 10, Dallimore Road, Manchester, England	Films for light micrographs and electron micrographs
Laboratory Thermal Equipment Ltd., Greenfield, Nr. Oldham, England	Tissue culture incubator
May and Baker Ltd., Laboratory Chemicals, Liverpool Road, Barton Moss, Eccles, Manchester	Sulphur free toluene
E. Merck, Darmstadt, West Germany	Giemsa and Azur II stains
Microflow Ltd., Fleet Mill, Minley Road, Aldershot, Hants., England	Laminar flow hood
Millipore Corporation, Bedford, Massachusetts, U.S.A. 01730	MF and Duralon filters
M.S.E. Scientific Instruments, Manor Royal, Crawley, Sussex, England	Centrifuges (Chilspin, Minor)
Nordic Immunological Laboratories Ltd., A.P. House, 2 St. Peters Road, Maidenhead, Berks., England	Conjugated rabbit anti-bovine IgG globulin
Oxoid Ltd., Wade Road, Basingstoke, Hants., England	Calcium and magnesium free phosphate buffered saline (Dulbecco A)
Packard Instrument Company, Subsidiary of Ambac Industries Inc., 2200 Warrenville, Downers Grove, Illinois, U.S.A.	Scintillation counter
Pye Unicam Ltd., York Street, Cambridge, England	Phillips 400 transmission electron microscope
Reichert-Jung, 820 Yeovil Road, Slough, England	Omu microtome

Rodwell Scientific Instruments, Rodwell-Bayne Ltd., 199-209 Hornchurch, Essex, England	Mackintosh Fildes jars
Shandon Southern Instruments Ltd., Chadwick Road, Astmoor, Runcorn, Cheshire, England	Cytocentrifuge (Cytospin)
Shering Corporation U.S.A. Distributed by Essex Chemie AG Lucerne, Switzerland	Gentamycin sulphate
Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo., U.S.A.	Bovine plasma albumin, polyvinylpyrrolidone, hypoxanthine, reduced glutathione, preservative free lithium heparin
Skatron AS, 3401 Lierbyen, Norway	Titertek cell harvester and papers
E.R. Squibb and Sons Inc., New York, N.Y., U.S.A.	Mycostatin (Nystatin)
Sterilin Ltd., 43-45 Broad Street, Teddington, England	Plastic universal and bijoux containers, disposable pipettes
TAAB Laboratory Equipment Ltd., 40 Grovelands Road, Reading, Berks., England	Reagents for electron microscopy
Union Carbide U.K. Ltd., Cryogenics Dept., Redworth Way, Aycliffe Industrial Estate, Darlington, Durham, England	Cryogenic containers
Whatman Ltd., Springfield Mill, Maidstone, Kent, England	Filter paper, glass fibre prefilters
Wheaton Scientific, Millville, New Jersey, U.S.A. 08332	Glass vials for cryopreservation
Winthrop Laboratories, Surbiton, Surrey, England	1% benzalkonium chloride (Roccal)

Appendix 2 Preparation of Reagents

Giemsa Stock Solution

10 g Giemsa powder (Merck)
540 ml glycerol (Analar grade - BDH Chemicals)
840 ml methanol (Solvent grade - BDH Chemicals)
2.76 g Azur II powder (Merck)

Grind Giemsa powder with a small volume of glycerol in a mortar with a pestle. Transfer to a conical flask, adding remainder of the glycerol. Incubate one hour at 60°C, shaking intermittently. Cool to room temperature, add methanol and mix with a magnetic stirrer overnight. Add Azur II powder and stir for 24-48 hours. Filter through a fluted No. 4 filter paper (Whatman) and store in dark bottles at room temperature.

Cacodylate Buffer (0.2M solution, pH 7.3)

Dissolve 4.28 g sodium cacodylate (Agar Aids) in
0.69 ml 1N hydrochloric acid (BDH Chemicals) and
distilled, deionized water (DDW), added to a final volume of 100 ml.

Dilute solution 1:1 with DDW and titrate to pH 7.3.

Glutaraldehyde Fixative (2.5%)

Mix 5 ml 25% glutaraldehyde solution (EM Scope Lab.)
25 ml 0.2M cacodylate buffer (pH 7.3) and
20 ml distilled, deionized water.

Add calcium chloride (Analar grade, BDH Chemicals) to a final fixative solution concentration of 2mM/ml.

Osmium Tetroxide Fixative (1%)

Dissolve 0.5 g osmium tetroxide (Johnson Matthey) in
20 ml 0.2M cacodylate buffer (pH 7.3) and
30 ml distilled, deionized water

Araldite

Mix 19 ml of a 1:1 solution of
araldite resin (EY 212, TAAB) and
DDSA hardener (HY 964, TAAB)

with 1 ml of a 1:4 solution of
DMP 30 accelerator (DY 064, TAAB) and
Di butyl phthalate (BDH Chemicals)

at 60°C for 10-15 minutes.

Scintillation Fluid

Dissolve 6 g 2,5 Diphenyloxazole (PPO) crystalline powder (D-4630, Sigma Chemical) and

0.05 g POPOP (P-3754, Sigma Chemical) in

1 litre sulphur free toluene (May and Baker)

Appendix 3 Theileria annulata (Hissar) cultures established with blood from calf 127 in Experiment 3.4
Parasitized erythrocyte (PRBC) counts arranged according to the number of parasites inside

Sample Day in vitro		Number of parasites in erythrocyte									PRBC per 1000 erythrocytes
		1	2	3	4	5	6	7	8	12	
	Blood smears										
0	a	96	4	0	0	0	0	0	0	0	14
	b	93	6	1	0	0	0	0	0	0	12
	Cultures										
2		97±2	3±2	0.4±0.7	0	0	0	0	0	0	11±2
4		64±15	2±1	2±2	30±9	0.2±0.5	0.1±0.3	0	0.4±0.6	0	9±2
6		38±10	2±2	7±7	50±10	0.3±0.6	0.5±0.8	0.04±0.2	0.7±0.8	0.04±0.2	11±2
8		41±12	7±3	10±6	41±10	0.2±0.5	0.2±0.4	0	0.5±0.6	0.5±0.6	6±3

Figures displayed are the mean values ± standard deviations based on counts of 100 PRBC and 1000 erythrocytes in 24 samples (i.e. pooled values of 2 wells sampled x 12 test factors).

Appendix 4 Theileria annulata (Hissar) cultures established with blood from calf 128 in Experiment 3.4
Parasitized erythrocyte (PRBC) counts arranged according to the number of parasites inside

Sample Day in vitro		Number of parasites in erythrocyte									PRBC per 1000 erythrocytes
		1	2	3	4	5	6	7	8	12	
	Blood smears										
0	a	93	6	1	0	0	0	0	0	0	14
	b	95	4	1	0	0	0	0	0	0	14
	Cultures										
2		96±2	4±2	0.3±0.5	0	0.04±0.2	0	0	0	0	13±4
4		87±6	2±1	0.4±0.6	10±5	0.2±0.5	0.04±0.2	0	0.2±0.6	0	11±3
6		59±10	3±2	4±3	34±9	0.2±0.4	0.2±0.4	0	0.3±0.6	0	9±2
8		68±16	4±2	4±3	24±15	0	0	0	0.1±0.4	0	13±3

Figures displayed are the mean values ± standard deviations based on counts of 100 PRBC and 1000 erythrocytes in 24 samples (i.e. pooled values of 2 wells sampled x 12 test factors).

Appendix 5 Theileria annulata (Hissar) in 127 cultures with different oxygen tensions, media and erythrocyte concentrations after 6 days in vitro

Counts of 100 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Culture treatment			12% Oxygen										19% Oxygen									
Erythrocyte concentration	Media	*Cultures sampled	Number of parasites in erythrocytes 1	2	3	4	5	6	7	8	12	Total parasites per 100 PRBC	Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	12	Total parasites per 100 PRBC
3%	M199	a	29	4	5	59	1	0	0	2	0	309	37	0	2	59	0	0	0	2	0	295
		b	30	5	5	58	0	0	0	1	1	307	43	3	1	53	0	0	0	0	0	264
	MEM-Alpha	a	25	5	17	52	0	0	0	1	0	302	33	1	2	64	0	0	0	0	0	297
		b	31	1	22	45	0	0	0	1	0	287	30	4	25	40	1	0	0	0	0	278
	RPMI 1640	a	49	6	0	33	0	2	0	0	0	205	44	1	9	44	2	0	0	0	0	259
		b	50	4	9	35	0	0	0	2	0	241	42	4	14	38	1	0	0	1	0	257
0.3%	M199	a	36	0	4	57	0	2	1	0	0	295	44	0	0	56	0	0	0	0	0	268
		b	43	2	3	51	0	0	0	1	0	268	51	1	2	44	0	1	0	1	0	249
	MEM-Alpha	a	20	5	12	61	0	2	0	0	0	322	30	2	5	62	0	1	0	0	0	303
		b	21	3	9	67	0	0	0	0	0	322	47	2	4	45	0	0	0	2	0	259
	RPMI 1640	a	35	2	7	54	0	2	0	0	0	288	55	0	4	39	1	0	0	1	0	236
		b	50	4	10	36	0	0	0	0	0	232	47	3	5	43	0	0	0	2	0	256

*2 cultures sampled per combination of test factors

Appendix 6 Theileria annulata (Hissar) in 128 cultures with different oxygen tensions, media and erythrocyte concentrations after 6 days in vitro

Counts of 100 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Culture treatment			12% Oxygen									19% Oxygen								
Erythrocyte concentration	Media	*Cultures sampled	Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	Total parasites per 100 PRBC	Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	Total parasites per 100 PRBC
3%	M199	a	54	0	1	45	0	0	0	0	237	67	0	0	33	0	0	0	0	199
		b	50	7	7	35	0	1	0	0	231	66	0	2	30	1	1	0	0	203
	MEM-Alpha	a	51	4	2	42	0	0	0	1	241	51	1	0	46	1	0	0	1	250
		b	42	9	7	40	0	0	0	2	257	59	6	3	30	1	1	0	0	211
	RPMI 1640	a	59	1	5	34	0	1	0	0	218	52	2	3	42	0	0	0	1	241
		b	45	5	11	38	1	0	0	0	245	60	4	1	35	0	0	0	0	211
0.3%	M199	a	65	4	0	31	0	0	0	0	197	55	2	1	41	0	1	0	0	232
		b	54	1	1	42	0	1	0	1	241	74	1	3	22	0	0	0	0	173
	MEM-Alpha	a	45	2	1	52	0	0	0	0	260	62	1	5	32	0	0	0	0	207
		b	57	2	1	39	0	0	0	1	228	55	1	8	36	0	0	0	0	225
	RPMI 1640	a	62	3	6	29	0	0	0	0	202	74	3	4	18	1	0	0	0	169
		b	82	0	2	16	0	0	0	0	152	78	2	3	17	0	0	0	0	159

*2 cultures sampled per combination of test factors

Appendix 7 Theileria annulata (Hissar) cultures established with blood from calf 145 in Experiment 3.5
Counts of parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day in vitro	Sample		Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	Total parasites per 100 PRBC	PRBC per 1000 erythrocytes
0	Blood smear		98	2	0	0	0	0	0	0	102	31
			96	4	0	0	0	0	0	0	104	20
6	M199/40 FBS	+BAE	26	1	5	63	1	2	0	2	328	16
			28	1	6	61	0	1	0	3	322	11
		-BAE	30	4	11	52	2	0	0	1	297	18
			23	8	11	55	0	2	0	1	312	18
	M199/20 FBS with hypoxanthine and glutathione	+BAE	38	0	4	57	1	0	0	0	283	16
			29	1	2	66	1	0	1	0	313	15
		-BAE	45	9	3	40	0	0	0	3	256	25
			30	2	5	59	1	1	0	2	312	22
	M199/20 FBS with hypoxanthine	+BAE	26	2	5	66	1	0	0	0	314	16
			27	3	4	64	0	1	1	0	314	19
		-BAE	34	2	1	61	1	0	0	1	298	21
			31	2	3	58	0	0	1	5	323	21
	M199/20 FBS with glucose	+BAE	35	4	3	57	1	0	0	0	285	20
			22	1	4	71	0	0	0	2	336	18
		-BAE	33	2	2	59	2	0	0	2	305	22
			24	5	1	70	0	0	0	0	317	19
	M199/20 FBS	+BAE	36	2	2	57	1	0	0	2	295	21
			30	1	3	59	1	2	0	4	326	17
		-BAE	40	4	2	53	0	0	0	1	274	19
			49	3	1	45	0	0	0	2	254	22

*2 cultures sampled per combination of test factors

Appendix 8 Theileria annulata (Hissar) cultures established with blood from calf 146 in Experiment 3.5
Counts of parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day in vitro	Sample		Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	Total parasites per 100 PRBC	PRBC per 1000 erythrocytes
0	Blood smear		94	6	0	0	0	0	0	0	106	30
			96	4	0	0	0	0	0	0	104	38
6	M199/40 FBS	+BAE	31	2	3	62	1	0	0	1	305	30
			35	2	4	58	0	0	0	1	291	30
		-BAE	35	5	17	40	2	1	0	0	272	39
			38	4	5	50	2	0	0	1	279	28
	M199/20 FBS with hypoxanthine and glutathione	+BAE	47	4	1	46	1	1	0	0	253	29
			40	6	3	49	0	0	0	2	273	23
		-BAE	75	6	4	13	2	0	0	0	161	38
			61	3	2	32	0	0	0	2	217	23
	M199/20 FBS with hypoxanthine	+BAE	72	1	2	25	0	0	0	0	180	38
			63	6	5	26	0	0	0	0	194	29
		-BAE	75	2	3	18	1	0	0	1	173	36
			61	3	0	35	1	0	0	0	212	35
	M199/20 FBS with glucose	+BAE	76	3	2	19	0	0	0	0	164	26
			72	2	3	22	1	0	0	0	178	35
		-BAE	70	2	1	25	0	0	0	2	193	27
			74	2	2	22	0	0	0	0	172	37
	M199/20 FBS	+BAE	47	4	1	46	1	1	0	0	253	29
			40	6	3	49	0	0	0	2	273	23
		-BAE	75	6	4	13	2	0	0	0	161	38
			61	3	2	32	0	0	0	2	217	23

*2 cultures sampled per combination of test factors

Appendix 9 Theileria annulata (Ankara) cultures in Experiment 3.6

Counts of parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

	Day in vitro	Sample	Number of parasites in erythrocyte 1	2	3	4	5	6	7	8	Total parasites per 100 PRBC	PRBC per 1000 erythrocytes
	0	Blood smears	96±1	4±1	0	0	0	0	0	0	104±1	22±3
		Cultures										
Calf 153	6	M199/40 FBS	52±13	2±1	4±2	40±12	0.5±1	1±1	0	0.2±0.4	238±39	23±6
		M199/20 FBS	59±8	3±3	3±2	34±4	0.3±1	0.3±1	0	1.1±1	219±22	24±5
		M199/40 NBS or AS	93±4	3±2	1±2	2±2	0	0	0	0	113±10	25±4
	0	Blood smears	94±1	5±1	1±0	0	0	0	0	0	107±1	87±7
		Cultures										
Calf 156	6	M199/40 FBS	54±14	3±1	2±1	35±13	1±1	0.1±0.4	0	0.3±0.5	216±40	85±16
		M199/20 FBS	88±4	6±3	1±0.5	5±7	0.2±0.4	0	0	0	123±18	80±12
		M199/40 NBS or AS	92±4	6±3	1±1	0.5±0.5	0	0	0	0	109±4	92±5

Figures are the mean ± standard deviations of pooled counts of parasites in 100 PRBC and number of PRBC per 1000 erythrocytes in 2 smears on day 0, and 6 culture samples after 6 days in vitro.

Appendix 10 Erythrocytes infected with Theileria annulata (Ankara) in blood from calf 154 on selected days after splenectomy

Counts of 500 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day after splenectomy	Number of parasites in erythrocyte					PRBC per 1000 erythrocytes
	1	2	3	4	5	
6	497	3	0	0	0	4
8	499	0	0	1	0	4
10	492	5	0	3	0	5
12	489	6	0	5	0	10
14	494	4	0	2	0	16
16	493	6	0	1	0	24
18	480	15	1	4	0	40
20	477	20	0	3	0	60
22	466	26	0	8	0	80
24	480	16	4	0	0	104
28	477	20	1	2	0	109
34	469	30	0	1	0	86
40	478	20	0	2	0	42

Appendix 11 Erythrocytes infected with Theileria annulata (Ankara) in blood from calf 155 on selected days after splenectomy

Counts of 500 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day after splenectomy	Number of parasites in erythrocyte					PRBC per 1000 erythrocytes
	1	2	3	4	5	
6	500	0	0	0	0	4
8	498	2	0	0	0	5
10	490	8	0	2	0	7
12	480	14	0	6	0	17
14	488	12	0	0	0	22
16	483	16	0	1	0	45
18	466	28	4	2	0	52
20	460	30	2	7	1	64
22	441	54	3	2	0	112
24	449	48	2	1	0	140
28	452	35	3	9	1	106
34	487	12	0	1	0	53
40	483	15	0	2	0	49

Appendix 12 Counts of erythrocytes infected with Theileria annulata (Ankara) observed (O) in blood from calf 154 on selected days after splenectomy compared to Poisson distribution (E)

Day after splenectomy		Number of parasites in erythrocyte 0	1	2	3	4	Total χ^2
6	O	996.0	3.98	0.024	0	0	0.03
	E	996.0	4.00	0.008	-	-	
8	O	996.0	3.99	0	0	0.008	5818.2*
	E	996.0	3.98	0.008	0.00001	0.00000001	
10	O	995.0	4.9	0.05	0	0.03	31034.6*
	E	994.9	5.1	0.01	0.00002	0.00000003	
12	O	990.0	9.8	0.12	0	0.01	20833.1*
	E	989.6	10.3	0.05	0.0002	0.0000005	
14	O	984.0	15.8	0.1	0	0.064	1333.2*
	E	983.8	15.7	0.1	0.0007	0.000003	
16	O	976.0	23.7	0.3	0	0.05	249.9*
	E	975.9	23.4	0.3	0.002	0.00001	
18	O	960.0	38.4	1.2	0.08	0.32	1024.1*
	E	958.6	40.5	0.9	0.01	0.0001	
20	O	940.0	57.2	2.4	0	0.36	215.5*
	E	938.5	59.6	1.9	0.04	0.0006	
22	O	920.0	74.6	4.2	0	1.30	843.0*
	E	915.8	80.6	3.5	0.1	0.002	
24	O	896.0	99.8	3.3	0.8	0	3.1
	E	896.7	97.7	5.3	0.2	-	
28	O	891.0	104.0	4.4	0.2	0.4	26.3*
	E	891.3	102.5	5.9	0.2	0.0065	
34	O	914.0	80.7	5.2	0	0.2	15.1*
	E	912.4	83.7	3.8	0.1	0.0027	
40	O	914.0	82.2	3.4	0	0.3	57.2*
	E	913.5	82.2	3.7	0.1	0.002	

Figures are the number of erythrocytes per 1000 with the specified number of parasites inside.

*Probability < 0.01

Appendix 13 Counts of erythrocytes infected with Theileria annulata (Ankara) observed (O) in blood from calf 155 on selected days after splenectomy compared to Poisson distribution (E)

Day after splenectomy		Number of parasites in erythrocyte 0	1	2	3	4	5	Total χ^2
6	O	996.0	4.00	0	0	0	0	0.0001
	E	996.0	3.98	-	-	-	-	
8	O	995.0	4.98	0.02	0	0	0	0.01
	E	995.0	4.99	0.01	-	-	-	
10	O	993.0	6.8	0.11	0	0.028	0	9000.1*
	E	992.8	7.1	0.03	0.00006	0.0000001	-	
12	O	983.0	16.3	0.5	0	0.2	0	10000.1*
	E	982.1	17.8	0.2	0.001	0.000004	-	
14	O	978.0	21.5	0.5	0	0	0	0.11
	E	977.7	22.0	0.2	-	-	-	
16	O	955.0	43.5	1.4	0	0.09	0	40.5*
	E	954.4	44.6	1.0	0.02	0.0002	-	
18	O	948.0	48.5	2.9	0.40	0.2	0	105.9*
	E	945.2	53.3	1.5	0.03	0.0004	-	
20	O	936.0	58.9	3.8	0.3	0.9	0.13	1655.8*
	E	930.9	66.6	2.3	0.06	0.001	0.00002	
22	O	888.0	98.8	12.1	0.7	0.4	0	20.8*
	E	880.9	111.7	7.1	0.3	0.01	-	
24	O	860.0	125.7	13.4	0.6	0.28	0	5.7
	E	856.1	133.0	10.3	0.5	0.02	-	
28	O	894.0	95.8	7.4	0.6	1.9	0.2	648.7*
	E	885.8	107.4	6.5	0.3	0.008	0.0002	
34	O	947.0	51.6	1.3	0	0.1	0	24.8*
	E	946.9	51.7	1.4	0.03	0.0004	-	
40	O	951.0	47.3	1.5	0	0.2	0	133.0*
	E	950.2	48.5	1.2	0.02	0.003	-	

Figures are the number of erythrocytes per 1000 with the specified number of parasites inside.

*Probability < 0.01

Appendix 14 Erythrocytes infected with Theileria annulata (Ankara) in blood from calf 163 on selected days after infection

Counts of 500 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day after infection	Number of parasites in erythrocyte												PRBC per 1000 erythrocytes
	1	2	3	4	5	6	7	8	9	10	11	12	
26	481	18	1	0	0	0	0	0	0	0	0	0	22
28	483	15	1	1	0	0	0	0	0	0	0	0	28
30	481	17	2	0	0	0	0	0	0	0	0	0	56
32	453	44	2	1	0	0	0	0	0	0	0	0	73
34	466	31	2	1	0	0	0	0	0	0	0	0	102
36	450	46	3	1	0	0	0	0	0	0	0	0	129
38	444	47	2	7	0	0	0	0	0	0	0	0	140
42	463	31	2	4	0	0	0	0	0	0	0	0	146
46	448	46	3	3	0	0	0	0	0	0	0	0	97
50	380	99	10	9	1	1	0	0	0	0	0	0	230
54	272	155	49	21	1	1	0	1	0	0	0	0	441
56	78	115	104	92	53	34	13	7	2	2	0	0	920

Appendix 15 Erythrocytes infected with Theileria annulata (Ankara) in blood from calf 164 on selected days after infection

Counts of 500 parasitized erythrocytes (PRBC) arranged according to the number of parasites inside

Day after infection	Number of parasites in erythrocyte												PRBC per 1000 erythrocytes
	1	2	3	4	5	6	7	8	9	10	11	12	
16	489	9	1	1	0	0	0	0	0	0	0	0	24
21	465	30	3	1	1	0	0	0	0	0	0	0	73
26	429	55	10	5	1	0	0	0	0	0	0	0	135
28	409	77	10	3	1	0	0	0	0	0	0	0	157
30	420	63	9	8	0	0	0	0	0	0	0	0	150
32	432	55	7	6	0	0	0	0	0	0	0	0	118
34	376	94	21	9	0	0	0	0	0	0	0	0	202
36	73	101	77	102	57	47	21	15	2	3	1	1	890
38	47	81	84	112	70	54	25	20	5	1	1	0	880

Appendix 16 Counts of erythrocytes infected with Theileria annulata (Ankara) observed (O) in blood from calf 163 on selected days after infection compared to Poisson distribution (E)

Day after infection		Number of parasites in erythrocyte											Total χ^2
		0	1	2	3	4	5	6	7	8	9	10	
26	O	978.0	21.0	0.8	0.090	0	0	0	0	0	0	0	4.8
	E	977.4	22.4	0.3	0.002	-	-	-	-	-	-	-	
28	O	972.0	27.0	0.8	0.06	0.06	0	0	0	0	0	0	121.1*
	E	971.3	28.3	0.4	0.004	0.00003	-	-	-	-	-	-	
30	O	944.0	53.9	1.9	0.2	0	0	0	0	0	0	0	1.1
	E	943.3	55.1	1.6	0.03	-	-	-	-	-	-	-	
32	O	927.0	66.1	6.4	0.3	0.14	0	0	0	0	0	0	14.7*
	E	922.7	74.2	3.0	0.1	0.002	-	-	-	-	-	-	
34	O	898.0	95.0	6.3	0.4	0.2	0	0	0	0	0	0	15.6*
	E	896.0	98.5	5.4	0.2	0.0025	-	-	-	-	-	-	
36	O	871.0	116.1	11.9	0.8	0.2	0	0	0	0	0	0	3.5
	E	866.6	124.1	8.9	0.4	0.02	-	-	-	-	-	-	
38	O	860.0	124.0	13.2	0.6	2.0	0	0	0	0	0	0	197.7*
	E	852.0	136.0	10.9	0.6	0.02	-	-	-	-	-	-	
42	O	854.0	135.2	9.0	0.6	1.2	0	0	0	0	0	0	70.7*
	E	852.0	135.0	12.8	0.6	0.02	-	-	-	-	-	-	
46	O	903.0	86.9	8.9	0.6	0.6	0	0	0	0	0	0	72.4*
	E	897.0	97.6	5.3	0.2	0.005	-	-	-	-	-	-	
50	O	770.0	174.8	45.5	4.6	4.2	0.46	0.46	0	0	0	0	340.2*
	E	739.9	222.7	33.5	3.4	0.3	0.02	0.0008	-	-	-	-	
54	O	559.1	239.8	136.7	43.2	18.5	0.9	0.9	0	0.9	0	0	490.4*
	E	480.0	352.0	129.0	32.0	5.8	0.9	0.1	0.01	0.002	-	-	
56	O	79.2	143.6	211.8	191.5	169.4	97.6	62.6	23.9	12.9	3.7	3.7	40.4*
	E	48.6	147.0	222.0	224.0	169.0	102.0	52.0	22.0	8.4	2.8	0.8	

Figures are the number of erythrocytes per 1000 with the specified number of parasites inside.

*Probability < 0.01

Appendix 17 Counts of erythrocytes infected with Theileria annulata (Ankara) observed (O) in blood from calf 164 on selected days after infection compared to Poisson distribution (E)

Day after infection		Number of parasites in erythrocyte													Total χ^2
		0	1	2	3	4	5	6	7	8	9	10	11	12	
16	O	976.0	23.5	0.4	0.05	0.05	0	0	0	0	0	0	0	0	125001.0*
	E	975.6	24.1	0.3	0.002	0.00000002	-	-	-	-	-	-	-	-	
21	O	927.0	67.9	4.4	0.4	0.2	0.2	0	0	0	0	0	0	0	2021.3*
	E	923.8	73.3	2.9	0.1	0.002	0.00002	-	-	-	-	-	-	-	
26	O	865.0	115.8	14.8	2.7	1.4	0.3	0	0	0	0	0	0	0	1232.2*
	E	851.8	136.6	11.0	0.6	0.02	0.00008	-	-	-	-	-	-	-	
28	O	843.0	128.4	24.2	3.1	0.9	0.3	0	0	0	0	0	0	0	78.8*
	E	825.7	158.1	15.1	1.0	0.04	0.002	-	-	-	-	-	-	-	
30	O	850.0	126.0	18.9	2.7	2.4	0	0	0	0	0	0	0	0	150.3*
	E	834.0	151.4	13.7	0.8	0.04	-	-	-	-	-	-	-	-	
32	O	882.0	102.0	13.0	1.6	1.4	0	0	0	0	0	0	0	0	202.4*
	E	870.6	120.6	8.4	0.4	0.01	-	-	-	-	-	-	-	-	
34	O	798.0	152.0	38.0	8.4	3.6	0	0	0	0	0	0	0	0	91.9*
	E	765.0	204.9	27.5	2.4	0.2	-	-	-	-	-	-	-	-	
36	O	110.3	129.9	179.7	137.0	181.5	101.4	83.6	37.4	26.7	3.6	5.3	1.8	1.8	315.8*
	E	393.0	127.2	205.8	222.1	179.7	116.3	62.8	29.0	11.7	4.2	1.3	0.4	0.1	
38	O	119.7	82.7	142.6	147.9	197.2	123.2	95.1	44.0	35.2	8.8	1.8	1.8	0	328.2*
	E	30.4	106.2	185.5	216.0	188.6	131.8	76.7	38.3	16.7	6.5	2.3	0.7	-	

Figures are the number of erythrocytes per 1000 with specified number of parasites inside

*Probability < 0.01

Appendix 18 Comparison between estimations and random counts of erythrocytes in Experiment 8.2

Random field number	Estimate	Count	Difference
1	250	235	+15
2	350	336	+14
3	300	299	+1
4	300	283	+17
5	200	150	+50
6	250	306	-56
7	275	282	-7
8	250	223	+27
9	275	297	-22
10	200	208	-8
11	250	236	+14
12	250	304	-54
Total	3,150	3,159	-9

Appendix 19 Liquid scintillation counts of Babesia bovis cultures after 24 hour incubation periods with (G-^{3}H) hypoxanthine or (2-^{3}H) adenosine*: Preliminary experiments

	Hypoxanthine		Adenosine	
	Incorporation Period I	Incorporation Period II	Incorporation Period I	Incorporation Period II
B. bovis (South Africa)	18.4 ± 1.9	18.6 ± 3.6	14.3 ± 4.0	18.6 ± 2.4
Control uninfected erythrocytes	1.6 ± 0.5	0.6 ± 0.7	1.4 ± 0.5	0.7 ± 0.3
B. bovis (Mexico)	19.9 ± 6.8	28.1 ± 8.7	11.0 ± 3.5	20.1 ± 4.4
Control uninfected erythrocytes	1.3 ± 0.3	2.0 ± 0.9	1.2 ± 0.3	1.5 ± 0.4

*Figures are mean values of six replicates ± standard deviations expressed as counts per minute x 10^3

Parasitaemias increased in cultures of both strains from 1-2% on day 0 to 4-5% during Period I and 7-9% in Period II.

Appendix 20 Liquid scintillation counts of Babesia bovis cultures after 24 hour incubation periods with tritiated nucleic acid precursors

Trial	Culture	Incorporation period	Purines (cpm x 10^3)				Pyrimidines (cpm x 10^2)		
			Hypoxanthine	Adenosine	Adenine	Guanosine	Uridine	Cytidine	Thymidine
1	B. bovis (South Africa)	I	13.1 ± 2.7	10.2 ± 2.4	2.4 ± 0.3	4.4 ± 0.7	3.0 ± 0.3	1.7 ± 0.8	0.8 ± 0.4
		II	16.8 ± 4.0	10.8 ± 3.3	6.4 ± 1.1	9.0 ± 1.7	9.0 ± 1.6	2.8 ± 1.8	0.6 ± 0.2
	B. bovis (Mexico)	I	11.3 ± 2.2	9.0 ± 0.7	2.0 ± 0.2	4.0 ± 0.9	1.6 ± 0.1	2.0 ± 1.6	1.0 ± 0.4
		II	17.8 ± 2.9	9.2 ± 1.3	5.1 ± 1.1	10.0 ± 1.8	11.0 ± 2.0	1.9 ± 0.6	0.6 ± 0.2
	Control uninfected erythrocytes	I	0.5 ± 0.2	0.7 ± 0.4	0.7 ± 0.1	0.3 ± 0.05	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
		II	0.6 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.4	0.7 ± 0.2	0.9 ± 0.4	0.4 ± 0.2
2	B. bovis (South Africa)	I	16.9 ± 3.8	8.2 ± 2.0	4.2 ± 0.9	6.6 ± 1.0	4.5 ± 0.6	1.5 ± 0.5	0.5 ± 0.1
		II	17.7 ± 2.5	12.1 ± 3.5	7.8 ± 2.5	9.6 ± 1.9	12.5 ± 3.9	2.6 ± 0.4	2.5 ± 2.2
	B. bovis (Mexico)	I	16.4 ± 1.8	8.2 ± 1.8	3.6 ± 0.9	6.0 ± 0.8	4.8 ± 0.8	1.5 ± 0.3	0.7 ± 0.5
		II	18.2 ± 2.4	11.9 ± 1.7	9.2 ± 1.5	10.2 ± 2.7	12.1 ± 0.8	3.1 ± 0.6	2.5 ± 1.8
	Control uninfected erythrocytes	I	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.2 ± 0.04	0.5 ± 0.1	0.7 ± 0.2	0.5 ± 0.2
		II	0.7 ± 0.5	0.9 ± 0.4	0.7 ± 0.1	0.3 ± 0.03	0.9 ± 0.3	0.8 ± 0.3	2.2 ± 1.7

*Figures are mean values of six replicates ± standard deviations expressed as counts per minute (cpm) x 10^3 or x 10^2

Appendix 21 Liquid scintillation counts of Babesia bovis cultures after incubation for 2, 6 or 24 hours Incubation with (G-^{3}H) hypoxanthine or (2-^{3}H) adenosine*

Culture	Hypoxanthine 2 hour	Hypoxanthine 6 hour	Hypoxanthine 24 hour	Adenosine 2 hour	Adenosine 6 hour	Adenosine 24 hour
B. bovis (South Africa)	6.0 ± 2.3	12.8 ± 1.5	17.7 ± 2.5	4.3 ± 0.3	10.5 ± 1.7	12.1 ± 3.5
B. bovis (Mexico)	6.7 ± 1.2	14.4 ± 2.3	18.2 ± 2.4	4.4 ± 0.6	11.0 ± 1.8	11.9 ± 1.7
Control uninfected erythrocytes	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.5	0.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.4

*Figures are mean values of six replicates ± standard deviations expressed as counts per minutes x 10^3

Isotopes were added at onset of Period II in Trial 2 with parasitaemias of 4-5%. At 2 and 6 hours parasitaemias were 4-6% and at 24 hours 7-8% in cultures of both strains.

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INCORPORATION OF TRITIATED NUCLEIC ACID PRECURSORS BY *BABESIA BOVIS IN VITRO*

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The incorporation of tritiated nucleic acid precursors, particularly hypoxanthine, has been used to quantitatively assess the effect of immune sera and antimalarial drugs on the growth of *Plasmodium falciparum in vitro*. Studies on some *Babesia* species in short-term cultures have provided an insight into the mechanisms of nucleic acid synthesis in these parasites but the uptake of radiolabelled nucleic acid precursors by *Babesia bovis* in continuous cultures has not been previously reported.

For comparative experiments, two strains of *B. bovis* were established in culture with blood from calves which had been infected either with a culture derived erythrocyte suspension of a Mexican strain or with cryopreserved blood originally from an infected calf in South Africa. The parasites have been grown continuously in stationary erythrocyte cultures by a modification of the Levy and Ristic method (1). The semi-automated microdilution technique used by Desjardins *et al.* (2) with *P. falciparum* was adapted for use with *B. bovis*. The uptake of the following tritiated nucleic acid precurosrs by *B. bovis* during different growth periods *in vitro* was assessed by liquid scintillation counting:
(G-^{3}H) Hypoxanthine; (2-^{3}H) Adenine; (2-^{3}H) Adenosine; (8-^{3}H) Guanosine; (5-^{3}H) Cytidine; (6-^{3}H) Thymidine; (6-^{3}H) Uridine.

The purine precursors, hypoxanthine, adenosine, adenine and guanosine, were readily incorporated by both strains of *B. bovis*. The uptake of adenosine and hypoxanthine was significantly greater than any of the other precursors. The incorporation of the pyrimidine precursors, uridine and cytidine, was minimal compared to the controls. Thymidine did not appear to be utilized at all by the parasites.

The potential applications of this test system for immunological studies or within a drug development programme will be discussed.

(1) Levy, M.G. and Ristic, M. (1980) Science *207*, 1218-1220.

(2) Desjardins, R.E., Carfield, C.J., Haynes, J.D. and Chulay, J.D. (1979) Antimicrobial Agents and Chemotherapy *16*, 710-718.

Financial support for this study was provided by the Marshall Aid Commemoration Commission.

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ELECTROPHORETIC STUDIES ON THE ISOENZYMES OF *BABESIA BOVIS*

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The electrophoretic separation of parasite-associated isoenzymes on starch-gel has been used in the characterisation of several closely related intraerythrocytic parasites, notably *Plasmodium, Theileria* and rodent *Babesia*. Similar studies on *Babesia* parasites infecting cattle, either isolated from infected blood or grown in continuous culture, have not been reported.

This paper reports preliminary observations on the isoenzyme patterns of two strains of *B. bovis* from Mexico and South Africa grown in microaerophilic stationary phase (MASP) cultures. Parasites were freed from the infected erythrocytes by ammonium chloride lysis, washed with PBS and lysed by freeze-thawing in the presence of a non-ionic detergent. After centrifugation lysates were applied on cotton strands to thin starch gels (mean thickness 0.9 mm) and examined for three parasite associated enzymes; Lactate dehydrogenase (LDH); Glucose phosphate isomerase (GPI) and NADP linked Glutamate dehydrogenase (GDH) previously reported as being present in rodent *Babesia* (Momen, 1979. Ann. trop. med. parasit., 73, 109).

The presence of these three enzymes was confirmed in both *B. bovis* lysates: the enzyme banding patterns of the two strains were similar.

Comparison of these results with similar electrophoretic studies on lysates prepared from the piroplasms of *Theileria parva* and *T. annulata* have revealed interesting differences between these closely related parasites. Unlike *B. bovis*, piroplasms of *Theileria* do not appear to have a parasite-associated LDH but their complex GPI patterns indicate variation at the species and strain level (Melrose and Brown, 1979. Res. vet. sci., 27, 379; Melrose *et al*. 1980, Res. vet. sci., 29, 298.

Further work is required in order to establish the value of isoenzyme electrophoresis as a taxonomic aid in identifying *Babesia* piroplasms.

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ISOELECTRIC FOCUSING USED TO DETECT THE SOLUBLE ENZYMES OF *BABESIA*

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Isoelectric focusing (IEF) has now emerged as a sensitive and rapid method for characterising proteins and has been used in taxonomic studies of some protozoal parasites. IEF has the highest resolution of any electrophoretic technique and will resolve components with isoelectric points that differ by 0.001 of a pH unit. Thus, theoretically, any genetic mutation that leads to the change of a single charged amino acid can be detected with this technique. The relationship of several species of *Babesia* was examined using this technique.

The material used in this study included erythrocytes collected from calves and from continuous cultures infected with two strains of *Babesia bovis*, originally from Mexico and South Africa. *B. rodhaini*, *B. muratovi* and *B. microti* were also obtained from infected mice. Soluble enzyme extracts were prepared from the parasitized material and uninfected control blood from the appropriate host species by a modification of the method used by Kahl *et al.* (1982). The soluble enzymes were analysed by IEF or agarose gels and stained for a number of isoenzymes including:
Glucosephosphate isomerase (E.C.5.3.1.9); Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49); Lactate dehydrogenase (E.C.1.1.1.27); Malate dehydrogenase (E.C.1.1.1.37); Isocitrate dehydrogenase (E.C.1.1.1.42); Esterase (E.C.3.1.1.1) Malic enzyme (E.C.1.1.1.40) and Glutamate dehydrogenase (E.C.1.4.1.3).

Using IEF plates with a pH range of 3-10 and staining for the enzymes PGI and ICD has detected differences between *B. bovis*, *B. rodhaini* and *B. muratovi*. Differences between the two strains of *B. bovis* have also been detected using LDH. Further amplification of these differences should be possible by the use of expanded range IEF plates.

Kahl, L.P., Anders, R.F., Callow, L.L., Rodwell, B.J. and Mitchell, G.F. (1982) International Journal of Parasitology *12*, 103-109.

Financial support for this study was provided by the Overseas Development Administration, London (R.B.) and the Marshall Aid Commemoration Commission (P.A.C.)

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DETECTION OF ANTIBODY TO *BABESIA BOVIS* USING A FLUORESCENT ANTIBODY STAINING TECHNIQUE WITH *IN VITRO* AND *IN VIVO* DERIVED ANTIGENS.

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Acetone-fixed, antigen smears of Mexican and South African strains of *Babesia bovis* were prepared with defibrinated, infected bovine blood and with aliquots of erythrocyte suspensions derived from continuous *B. bovis* cultures (1) Each antigen preparation was tested for its reactivity using a fluorescent antibody staining technique with sera collected from calves infected with each strain of *B. bovis*.

Each of the antigen preparations detected antibodies to *B. bovis* in calves infected with both strains of the parasite. The *in vitro* derived antigen preparations first detected antibody to *B. bovis* 7 days after infection whereas *in vivo* derived antigen preparations did not detect antibodies until 12 days after infection. In the case of the *in vitro* derived antigens the sera collected 7-10 days after infection stained the parasites alone while sera collected from 12 days after infection stained both parasite and infected erythrocyte. The fluorescence seen with the *in vivo* derived antigen preparation was confined to the infected erythrocyte. Antigen smears prepared from concentrated, *in vitro* derived *B. bovis* infected erythrocytes which had been separated from non-infected erythrocytes as described by Kahl *et al.* (2) showed a marked reduction in the intensity of the parasite fluorescence and an increasingly granular erythrocyte fluorescence.

The results from these studies have confirmed the suitability of *in vitro* derived *B. bovis* as a source of antigen for immunological studies on *B. bovis* infection. They also indicate, however, that attempts to "purify" antigens in such preparations should be approached with caution as some methods e.g. hypotonic lysis may result in the loss of some antigens.

(1) Levy, M.G. & Ristic, M. (1980), Science 207, 1218-1220.

(2) Kahl, L.P., Anders, R.F., Callow, L.L., Rodwell, B.J. & Mitchell, G.F. (1982) International Journal for Parasitology 12, 103-109.

Financial support for this study was provided for by the Overseas Development Administration, London (TWJ) and the Marshall Aid Commemoration Commission (PAC)

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SEROLOGICAL EVIDENCE FOR THE EXISTENCE OF *B. BOVIS* INFECTION IN CATTLE IN BELIZE, CENTRAL AMERICA

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180 serum samples collected from adult cattle on seven farms in different areas of northern Belize were tested for the presence of *B. bovis* antibody using a fluorescent antibody staining technique employing acetone fixed, *in vitro* derived smears of *B. bovis* infected erythrocytes as antigen. 97% of these serum samples were shown to be serologically positive for *B. bovis* antibody when tested at a dilution of 1:50. The majority of positive sera stained the parasite and the infected erythrocyte, but some sera stained the parasite alone. The proportion of sera staining the parasite alone varied from 0-5% according to the farm. Sera collected from animals experimentally infected with *B. bigemina* and *B. divergens* did not react with *B. bovis* antigen preparations at dilutions greater than 1:40.

These results indicate that *B. bovis* infections of cattle is endemic over a wide area of northern Belize and the low reported incidence of clinical babesiosis suggests the existence of an enzootically stable situation in this region.

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Short Communications

The use of Eriochrome* Black A (diamond black) as a counterstain in fluorescent antibody staining techniques for the detection of antibodies to haemoprotozoa

The sensitivity and specificity of fluorescent antibody staining techniques for the detection of antibodies to a variety of haemoprotozoan parasites is well established (Nairn, 1976). A disadvantage of these techniques for the quantitative study of antibody concentrations is the subjective nature by which end-point titres are established, due to the diminishing degree of contrast between the organism and the background as the end-point is approached. Furthermore, some antigen preparations, such as isolated organisms, may lack any appreciable background fluorescence, making the identification of unstained areas difficult. Counter-staining with a fluorescent dye will heighten the contrast between specific staining and background staining (Goldman, 1968), but few such dyes are in general use, probably due to the suspicion that counter-staining reduces the intensity of the specific fluorescence. We have tested one of the 2,2′-dihydroxyazo dyes listed by Hall and Hansen (1962) for its suitability as a counterstain for FITC-stained preparations of several haemoprotozoan parasites as, in our experience, the commonly used counterstains (Evans Blue, Congo Red and rhodamine-labelled bovine albumin) do not impart sufficient colour to such antigen preparations. The dye selected, Eriochrome* Black A (obtained as diamond black, colour index 15710; BDH Ltd., Poole), was prepared as described in Goldman (1968) except that the initial (unchelated) dye solution consisted of 10 mg diamond black dissolved in 1 ml N,N-dimethylformamide. Immediately before use the chelated dye was diluted × 10 in PBS pH 7·2.

Antigen preparations consisting of acetone-fixed, thin smears of *Trypanosoma evansi*, *Babesia rodhaini* and *Theileria annulata*, were prepared from either infected mouse blood (*T. evansi* and *B. rodhaini*), infected bovine blood (*T. annulata* piroplasm antigen) or an *in vitro* culture suspension of bovine lymphocytes infected with *T. annulata* macroschizonts. Antisera to *T. evansi* and *B. rodhaini* were obtained from mice infected with the respective parasite and antisera to *T. annulata* from infected cattle. Each serum was titrated for antibody activity against its respective antigen as described in Hickerton and Jones (1981). Antigen/antibody complexes were visualized with an appropriate FITC-labelled anti-species IgG conjugate (RAM/FITC or RAB/FITC, Nordic Immunological Laboratories, Maidenhead). After the final wash in PBS the antigen preparations were overlaid with diluted diamond black chelate for five minutes, drained, washed in PBS for five minutes and mounted in 66% glycerol in 50 mM Tris, pH 9·0. Preparations were examined for fluorescence with an Orthoplan Microscope (Leitz, Wetzler, Germany) equipped for selective, incident excitation of FITC. The fluorescence was observed through a K530 edge filter at a total magnification of × 675.

Counterstaining of each of the antigen preparations with diamond black increased the contrast between the specific (parasite) fluorescence and the background without either a decrease in the intensity of the specific fluorescence or alteration in the end-point titre. The parasites appeared as green stained entities against a red stained background of host blood cells in the antigen smears prepared from infected blood. In the case of *T. annulata* macroschizont antigen the parasite appeared as a discrete green area within the red stained lymphocyte. The intensity of the specific (green) fluorescence in each of the antigen

*Eriochrome is a registered trade mark of CIBA-Geigy Ltd.

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preparations decreased towards the end-point, at which point there was a sharp transition from green to red stained parasites.

Counterstaining of haemoprotozoal antigen preparations with diamond black after labelling with specific antibody, therefore, greatly facilitated the differentiation of the parasite specific fluorescence from the background fluorescence, especially at low antibody concentrations. Such counterstaining has also proved to be of use in studies on the location of specific antigens in tissue sections (G. H. K. Lawson 1982, personal communication) and may be of use in topographical studies on the antigenic determinants of single cells as recognized by monoclonal antibodies.

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